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Identification of Horses with Resistance to Small Strongyles

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by Glen Coleman, Jennifer Seddon, & Kim Jell

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Foreword

Small strongyles, or cyathostomins, are important to the horse industry both as agents of disease (such as colic and clinical larval cyathostominosis) and because of widespread anthelmintic resistance. The horse industry relies heavily on macrocyclic lactone (ML) products for cyathostomin control and this is cause for considerable concern, as it is likely to select for ML-resistant strains of parasite. Some scientists have expressed concern that intensive ML use may harm the environment through their potential toxicity against dung fauna. Many horse owners/managers currently rely on an interval worm control program where all horses are treated at fixed periods of time (that varies with the product being used). The cost-effectiveness of this is questionable and it is likely that, on many properties, anthelmintics are being used excessively. This project is relevant to all sectors of the horse industry in Australia, both recreational and commercial, because cyathostomin infection of horses is widespread, and because of the heavy usage of anthelmintics by many horse owners.

The project is a pilot study that investigates the feasibility of developing a simple procedure (or procedures) for the identification of an individual horse's resistance to cyathostomin infections and/or contribution to pasture contamination with nematodes. Such a test would facilitate the development of more rational nematode control strategies by identifying those animals that may usefully be targeted for more regular anthelmintic treatments. This offers significant economic advantages (through less anthelmintic usage) as well as welfare benefits (by prolonging the useful life of the only remaining effective anthelmintic group) and, potentially, environmental benefits to the horse industry.

Evidence was collected that supports the hypothesis that a small number of horses contribute disproportionately fewer eggs to pasture contamination with cyathostomins. If these horses could be reliably identified with a single test, they could be treated less regularly with anthelmintics than the remainder of the herd and this would lead to cost savings and a reduction in selection pressure for anthelmintic resistance in cyathostomin populations on these properties. Conversely if horses could be identified that regularly contribute disproportionately more eggs to pasture contamination, then these animals could be the focus of worm control strategies. This study found that stallions and ponies were likely to contaminate pastures more heavily (i.e. have higher faecal egg count; [FEC]) than geldings/mares or other breeds respectively, suggesting that this needs to be factored into the design of integrated worm control programs for horse properties.

The study found little correlation between luminal worm burden and FEC, although FEC still reflects a horse's contamination of pasture with parasites. Serum from some horses possesses an agent with anthelmintic activity as measured in *in vitro* larval migration assays, but it appears unlikely that the anthelmintic activity present in the serum is correlated with ability to withstand worm burdens or FEC. However, further work, including with younger animals, is required to confirm this. There was some evidence that MHC haplotype may be correlated with consistently lower FEC, but repeating this work using larger sample sizes is required to confirm this.

Further investigations into the relationships between levels of pasture contamination, serum anthelmintic activity and MHC haplotype are warranted.

This project was funded from industry revenue which is matched by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1800 research publications, forms part of our Horse R&D Program, which aims to assist in developing the Australian horse industry, and enhancing its export potential.

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Peter O'Brien
Managing Director
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Abbreviations

BZ	Benzimidazole
CI	Confidence Intervals
EPG	Eggs Per Gram
FEC	Faecal Egg Count
FECRT	Faecal Egg Count Reduction Test
ICC	Inter-class Correlation
L3	Third Stage Larvae
LMI	Larval Motility Inhibition
MHC	Major Histocompatability Complex
ML	Macrocyclic Lactone
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
SSCP	Single Strand Conformation Polymorphism

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What the report is about

The project investigates the feasibility of developing a simple test (or tests) for the identification of an individual horse's resistance to cyathostomin infections and/or contribution to pasture contamination with nematodes. Such a test would facilitate the development of more rational nematode control strategies by identifying those animals that may usefully be targeted for more (or less) regular anthelmintic treatments.

Who is the report targeted at?

This project is relevant to all sectors of the industry in Australia, both recreational and commercial, because cyathostomin infection of horses is widespread, and because of the heavy usage of anthelmintics by many horse owners.

Background

Over the last twenty years, small strongyles or cyathostomins have emerged as the most important internal parasites of the horse. The significance of cyathostomins to the horse industry lies in both their pathogenicity (as agents of colic and/or clinical larval cyathostominosis) and in their resistance to anthelmintics. Many equine properties continue to rely on interval treatment programs (e.g. every six weeks for a benzimidazole product, or every ten weeks for ivermectin) for nematode control in horses. This approach to worm control is expensive and not sustainable in the long term, as relying almost exclusively on anthelmintic treatment of stock for worm control results in greater selection pressure for resistance.

Better targeting of anthelmintic treatments to individual animals offers significant economic advantages (through less anthelmintic usage) as well as welfare benefits (by prolonging the useful life of the only remaining effective anthelmintic group) and, potentially, environmental benefits.

Aims/objectives

To test the hypothesis that a small number of individual horses within a herd consistently harbour larger cyathostomin burdens and/or contribute disproportionately to pasture contamination with these parasites.

To investigate the feasibility of developing a simple test (or combination of tests) for the identification of an individual horse's resistance to cyathostomin infection and/or its likelihood of contributing disproportionately to pasture contamination with worms. This was achieved by means of an abattoir survey to identify horses with high and low cyathostomin burdens and investigation of levels of correlation between worm burdens and:

- faecal egg count (FEC)
- ability of serum to inhibit cyathostomin larval motility
- MHC haplotype.

Methods used

A prospective study of 54 horses on nine south-east Queensland properties was undertaken to determine if individual animals within a herd consistently contribute disproportionately to pasture contamination with cyathostomins. A range of potential explanatory variables for FEC values were examined.

An abattoir survey was used to determine the strength of association between FEC and cyathostomin populations. A larval migration inhibition assay was used to study the effects of various horses' sera in inhibiting the migration of cyathostomin larvae. Sera from horses that consistently had higher counts in the prospective study were compared with sera from animals that consistently had lower counts. The haploptypes of two Major Histocompatibility Complex (MHC) genes, *DQA* and *DRB*, were analysed in 55 horses, and the relationship between FEC and MHC haplotype examined.

Results/key findings

Data from the prospective trial supported the hypothesis that a small number of horses contribute disproportionately less to pasture contamination with cyathostomins, suggesting it is likely that the converse is also true (i.e. that a small number of animals contribute disproportionately more).

FEC were poorly correlated with luminal cyathostomin burdens; in fact, there was evidence that these nematodes exhibit a population density-dependent effect on worm fecundity.

Although horse serum does inhibit the migratory ability of cyathostomin larvae, this activity appears to be unrelated to the luminal strongyle burdens or FEC of the horse from which the serum was collected. Further work is required to confirm this finding.

No association between heterozygosity or specific alleles at the DQA locus and FEC could be demonstrated in the samples examined. However there was evidence that some DRB-linked microsatellite alleles were associated with low FEC and hence with low contamination of pastures.

Additional findings included observations that stallions were likely to have higher FEC than geldings or mares, and that ponies were likely to have higher FEC than the other breed groups examined.

Implications for relevant stakeholders:

The demonstration that certain horses within a group consistently contribute fewer eggs to contaminate pastures than their herd-mates, and the likelihood that other individuals contribute disproportionately more to pasture contamination, offers an opportunity for the industry to better target anthelmintic usage. This will have cost savings in the use of drugs, and may also slow the development of anthelmintic resistance.

A practicable test to identify likely levels of pasture contamination by an individual horse is required to facilitate the development of these strategies. On the basis of this work, further evaluation of alleles at the DRB locus and their relationship to FEC is desirable. While less promising, further evaluation of the ability of an individual horse's serum to inhibit cyathostomin larval migration is also warranted. The study was not able to identify markers that reflected luminal worm burdens of horses.

An unexpected finding was the evidence gathered that cyathostomin fecundity was influenced by worm density. The significance of this for the industry is two-fold:

- it complicates the interpretation of results from the Faecal Egg Count Reduction Test (FECRT), the standard test for anthelmintic resistance in the horse. Worms that survive a drug treatment are likely to significantly increase their egg output. This may lead to an underestimation of drug efficacy based on a comparison of pre- and post- drug FECs.

- it may facilitate the development of anthelmintic resistance, because following treatment there is a boost in the fecundity of worms that have survived the treatment (i.e. those with some degree of resistance to the drug).

Recommendations

- This study identified potential tools to identify horses contributing disproportionately to pasture contamination with cyathostomin larvae. However it was not able to identify a tool to easily identify horses with lower (or higher) worm burdens *per se*. Further work on the immune response of horses to cyathostomin infections is warranted, as it will likely facilitate the development of more strategic approaches to worm control in the horse.

- Further investigations of the apparent population density-dependent effects on fecundity are clearly warranted, as this phenomenon may impact both on the development of drug resistance and its detection (via the FECRT).

- Further analysis of the relationship between DRB alleles and FEC is warranted, as this gene is the most promising candidate for a simple test to determine whether an individual horse is likely to consistently shed fewer or more worm eggs onto pastures than other animals in its group.

- In addition, further evaluation of the use of a horse's serum in a cyathostomin larval migration inhibition assay is also warranted.

- When monitoring levels of pasture contamination, horse owners ought to be aware that stallions and pony breeds are likely to have higher FEC than geldings, mares or other breeds.

1. Introduction

1.1 Anthelmintic resistance and the targeting of anthelmintic treatments

Over the last twenty years, small strongyles or cyathostomins have emerged as the most important internal parasites of the horse. The significance of cyathostomins to the horse industry lies in both their pathogenicity (as agents of colic and/or clinical larval cyathostominosis) and in their resistance to anthelmintics (Love, Murphy et al. 1999; Pook, Power et al. 2002). Resistance to benzimidazoles is very widespread and pyrantel resistance has been reported on a number of occasions (Lyons, Tolliver et al. 1999; Pook, Power et al. 2002). The growing reliance of the horse industry on macrocyclic lactone (ML)-based products such as ivermectin or milbemycin is cause for considerable concern, as ML-resistance in cyathostomins is likely inevitable if current practice continues (Kaplan 2004). Indeed, a report from German scientists provided the strongest evidence yet for ivermectin-resistance in these nematodes (Samson-Himmelstjern G von, Fritzen B et al. 2005).

Many equine properties continue to rely on interval treatment programs (e.g. every six weeks for a benzimidazole product, or every ten weeks for ivermectin) for nematode control in horses (Osterman Lind, Uggla et al. 2005). This approach to worm control is expensive and not sustainable in the long term, as relying almost exclusively on anthelmintic treatment of stock for worm control results in greater selection pressure for resistance (Leathwick, Pomroy et al. 2001). Many of the most innovative new approaches to nematode control have arisen within the small ruminant industries, where anthelmintic resistance has reached near crisis point (Kaplan 2004). For example, an approach that has received considerable attention in small ruminant industries is to confine treatment to animals suffering from parasitism so that only a relatively small proportion of the total parasite population is exposed to drugs at any one treatment (Wolstenholme, Fairweather et al. 2004).

Selection pressure for resistance is affected by the proportion of the nematode population in refugia, or not exposed to anthelmintic (Van Wyk 2001). Overdispersion of parasites in hosts is well known, and means that a relatively small proportion of the host population harbours the majority of the parasite population (Anderson and May 1985). This is a reflection of the variation in levels of acquired immunity between individual animals. In sheep the most resistant 50% of the flock produce less than 10% of the worm eggs, whereas the most susceptible 15% of animals produce 50% of the eggs (Kloosterman, Parmentier et al. 1992). In cattle the figures are reported to be less extreme, with the most susceptible 25% of animals producing about half the worm eggs (Kloosterman, Parmentier et al. 1992).

The extent of this phenomenon in equine cyathostomin infections has received little attention, although it is assumed to occur. There is clear evidence that acquired immunity develops against cyathostomins in horses, but the extent to which it varies between individuals is unclear (Klei and Chapman 1999). The prospective study proposed in this application aims to quantify whether certain animals contribute disproportionately to levels of pasture contamination with cyathostomin eggs/larvae (L3s).

If particular horses can be shown to consistently pass higher numbers of eggs onto pastures, then a method for the identification of such animals would allow the horse owner/manager to target anthelmintic therapy to these horses only. This would decrease selection pressure for resistance by maintaining a significant proportion of the cyathostomin population in refugia (in untreated horses, and as L3s on the pasture). It could also lead to significant cost savings via decreased use of anthelmintic compounds. The cost savings would only be apparent however if a simple, inexpensive test was available to distinguish highly resistant horses from least resistant horses.

1.2 Possible approaches to the identification of resistant horses

Faecal egg counts (FEC) offer one approach to the identification of resistant or susceptible animals in a population, and have been advocated in the sheep industry as one component of a selection index for the identification of resistant animals for breeding (Bisset, Morris et al. 2001). Although correlation between FEC and cyathostomin burdens is likely to be variable (being influenced by factors such as the numbers of immature worms and decreased worm fecundity in immune animals), this has not been examined in detail and FEC is often cited as indirect evidence of levels of immunity against cyathostomins (Klei and Chapman 1999). The FEC requires fresh faeces (preferably direct from the rectum), is laborious to perform (and therefore expensive) if large numbers of horses are involved, and needs to be accompanied by larval culture to identify any contribution to FEC from large strongyles.

In cattle, parasite-specific IgG antibodies in serum are the most widely used marker to evaluate levels of acquired immunity (Claerebout and Vercruysse 2000). Similarly, Dowdall and colleagues at the Faculty of Veterinary Science, University of Liverpool, have purified a 20 kDa antigen complex from somatic mucosal cyathostomin larvae. Serum IgG(T) responses to this complex were correlated with luminal cyathostomin populations (Dowdall, Proudman et al. 2004). The Liverpool group's primary interest is in the development of a diagnostic assay for the clinical condition of larval cyathostominosis, but there is an opportunity for future collaboration with this group in refining this serological assay for use in identifying resistant horses.

A third approach to the identification of horses with resistance to cyathostomins is offered by the work with cattle of Claerebout et al. (1999). This group demonstrated that serum from *Ostertagia*-infected cattle inhibited larval motility and migration. Serum larval motility inhibition (LMI) was significantly negatively correlated with *Ostertagia* worm counts, and sera from more immune animals exhibited higher LMI activity. Larval motility assays are attracting increasing interest in evaluating anthelmintic resistance, so it would be relatively simple to adapt one of these assays to evaluate sera from individual horses to assess resistance status or level of infection.

1.3 The potential for identifying genetic markers of resistant horses

The most important problem with each of the techniques described above is that results are likely to be influenced by the level of exposure of the animal or herd to cyathostomin infection. Comparison of results from different properties, or even different times on the one property, will thus be extremely difficult. Genetic markers of resistance offer the promise of not being influenced by levels of exposure to parasites, as well as simpler sampling techniques (blood or hair only needs to be collected). Considerable effort has gone into the identification of genetic markers of resistance in sheep (Bisset, Morris et al. 2001), but the ovine research is constrained by the need to identify a marker that will be useful in breeding programs (e.g. one that is not linked with undesirable production traits). It is important to note that the intention in this project is simply identification of a marker that will help the industry identify horses that will require more (or less) frequent anthelmintic treatment.

Resistance to parasite infection has been demonstrated in other species to be moderately heritable, with most of this work being conducted in sheep. The heritability of FEC varied from 0.2 at weaning to 0.65 at 400 days in merinos (Pollott, Karlsson et al. 2004), and an average heritability of strongyle FEC of 0.23 was found in Scottish blackface sheep (Davies, Stear et al. 2006). It is reasonable to hypothesise that similar values would be found in the horse. These levels of heritability suggest there is a substantial genetic component to account for the variation in host resistance to parasites.

One of the gene families implicated in resistance to endoparasite infections is the major histocompatibility complex (MHC) group of genes. A microarray analysis compared the expression pattern of genes between lines of sheep selected for high and low FEC (Diez-Tascom, Keane et al. 2005). Of the 28 unique genes identified, immune-related genes were a predominant category, including MHC genes. Another study searching for quantitative trait loci (QTL) to use in a marker-

assisted selection scheme in Scottish blackface sheep identified regions close to the MHC that showed significant association with non-*Nematodirus* strongyle FEC traits (Davies, Stear et al. 2006). Particular MHC alleles have been associated previously with either an increased or a decreased resistance to nematodes in sheep (Paterson, Wilson et al. 1998). For example, one of the class II MHC genes, *DRB1*, was reported responsible for 14% of the phenotypic variation in FEC in Suffolk sheep (Sayers, Good et al. 2005). This effect holds across species, with particular *DRB1* alleles found more commonly in individuals with high FEC, for example, in the striped mouse in the Southern Kalahari (Froeschke and Sommer 2005). However, no information is available for the horse and, while significant associations have been found in other species, associations are not universal (for example, (Cooper, van Oorschot et al. 1989), emphasising the desirability of exploring this issue in the horse.

1.4 Conclusions

The horse industry in Australia is worth more than $8 billion per year and includes approximately 1.2 million horses. This project will benefit all sectors of the industry in Australia, both recreational and commercial, because cyathostomin infection of horses is widespread, and because of the heavy usage of anthelmintics by many horse owners.

This project is a pilot study that investigates the feasibility of developing a simple procedure (or procedures) for the identification of an individual horse's resistance to cyathostomin infections, and/or its contribution to pasture contamination with nematodes. Such a test would facilitate the development of more rational nematode control strategies by identifying those animals that may usefully be targeted for more regular anthelmintic treatments. This offers significant economic advantages (through less anthelmintic usage) as well as welfare benefits (by prolonging the useful life of the only remaining effective anthelmintic group) and, potentially, environmental benefits to the horse industry.

2. Objectives

To test the hypothesis that a small number of individual horses within a herd consistently harbour larger cyathostomin burdens and/or contribute disproportionately to pasture contamination with these parasites.

To investigate the feasibility of developing a simple test (or combination of tests) for the identification of an individual horse's resistance to cyathostomin infection, and/or its likelihood of contributing disproportionately to pasture contamination with worms. This was achieved by means of an abattoir survey (to identify horses with high and low cyathostomin burdens) and the prospective survey and investigation of levels of correlation between worm burdens and faecal egg count, ability of serum to inhibit cyathostomin larval motility, and MHC haplotype.

3. Methodology

3.1 Prospective study

The aim of the prospective study was to test the hypothesis that a small number of individual horses within a herd consistently harbour larger cyathostomin burdens, and/or contribute disproportionately to pasture contamination with these parasites. Horses were assigned to high, low and intermediate parasite burden groups for further experimental work.

Nine multi-horse properties in south-east Queensland were visited monthly over an 18 month period commencing in January 2007 (with a period of interruption on most properties due to an outbreak of equine influenza). Freshly-passed faeces were collected from each animal (a total of 54 horses) at each visit. Data on age, sex, and reproductive status of each animal was collected, as was information on anthelmintic use on the property (dates, method of administration, product used). FEC and larval culture (to identify proportion of eggs attributable to cyathostomins) was performed on each sample using standard techniques. Hair and blood samples were also collected from each animal and stored for use in the molecular work outlined below.

Each horse was classified on the basis of:

- individual animal identification code
- age; as ≤5 years or > 5 years, on the assumption that horses over five years of age were likely, in most circumstances, to have developed levels of acquired immunity reflecting their genetic resistance/susceptibility to infection
- sex; mare/gelding versus stallion, based on evidence that entire males in other species express lower levels of immunity to parasites
- breed or type; quarter-horse/quarter-horse crosses/stockhorses/thoroughbreds versus 'warmbloods' versus Arabs versus ponies, and
- property on which it was housed.

Faecal samples were classified by season (summer - December, January, February; autumn - March, April, May; winter – June, July, August; spring – September, October, November) and whether or not the horse had received anthelmintic treatment the previous month and two months (classified as none, benzimidazole (BZ), macrocyclic lactone (ML)).

Property owners were requested to treat their horses only when the mean FEC for all horses on the property was ≥ 200 eggs per gram (EPG).

3.1.1 Zero-inflated negative binomial

As the FEC data were heavily skewed and contained a high proportion of zero counts (48.5%), a zero-inflated negative binomial model was used to assess the associations between explanatory variables and FEC. Explanatory variables were fitted separately in univariable models in both the negative binomial and logistic parts of the model. Selected variables were progressively included in a multivariable model; variables were retained in the final model when the likelihood ratio test returned a p-value < 0.05. 95% confidence intervals (CI) were calculated using the Huber/White estimate of variance to account for clustering of FEC within horses. All analyses were undertaken using Stata Version 10 (StataCorp, College Station, TX, USA, 2007).

3.1.2 Logistic regression for estimation of ICC

To estimate the within-horse correlation in FEC, only FECs from horses that had not received macrocyclic lactone treatment for at least three months were included. This data set was included to allow for the suppression of FEC following treatment with macrocyclic lactone products for a mimimum of 8-10 weeks, compared with only four to five weeks for BZ products (Herd and Gabel 1990; Eysker, Bakker et al. 2008). All FECs from period 1 were removed as it was not known whether a macrocyclic lactone had been used three months previously. Horses for which only one FEC remained were removed from the dataset. As it is currently not possible to estimate intraclass correlation co-efficients for data with a negative binomial distribution, FECs were recoded to dichotomous outcome variables – FEC$\geq$=1, FEC$\geq$=50, FEC$\geq$=100, FEC$\geq$=200, FEC$\geq$=500. A random effects logistic regression model was then fitted for each of these outcome variables (using the xtlogit function), with horse identification as the random effect. The null model was fitted initially then each of the following explanatory variables were fitted in univariable models to obtain estimates of the intraclass correlation co-efficient: age, breed, sex, property and season. All analyses were undertaken using Stata Version 10 (StataCorp, College Station, TX, USA, 2007).

3.2 Abattoir survey

The aim of the abattoir survey was to determine the strength of correlation between cyathostomin populations and FEC. Horses were assigned to high, low and intermediate parasite burden groups for further experimental work.

Large intestines, caecum, faeces, blood (smears, whole blood and serum), and hair was collected from horses at a local horse knackery (horses are grazed on the property for variable periods of time before slaughter, and it is known that the majority of animals have not received recent anthelmintic treatment). Data was collected on age and sex of each animal, as well as time of year when animal was slaughtered. Horses sampled were between two and 20 years of age (assessed via dental wear patterns).

Using previously published sampling techniques (Bucknell, Gasser et al. 1995), the luminal (L4 and adult) populations were calculated for each animal. Five hundred luminal worms from each section of the large intestinal tract (caecum, dorsal and ventral colon) were preserved for later identification as part of a separate study. Current recommendations are to identify up to 1500 worms per horse if the aim is to detect the presence of rarer species of cyathostomin (Chapman, Kearney et al. 2003). In addition, FEC (coupled with larval culture and identification of infective larvae, or L3s) was conducted on each animal using a standard modified McMaster technique. Correlations between FEC and luminal cyathostomin populations were evaluated using standard statistical techniques.

3.3 Larval migration inhibition activity

The aims of this study were to assess levels of nematode larval migration inhibition of equine sera and compare variation of inhibitory activity between animals that consistently shed high (or low) numbers of cyathostomin eggs.

A modification of the technique used by Claerebout, Agneessens et al. (1999) was used. Faeces from horses that had not been treated with an anthelmintic in the previous eight weeks were cultured and cyathostomin L3s recovered. The sera used in the assay were collected from animals in the prospective study; sera from animals that had consistently low FEC compared to other animals on the same property were compared to animals that had consistently higher FEC.

The ability of horse serum to inhibit motility of cyathostomin larvae was assessed by placing exsheathed third stage larvae (approximately 50 L3s per well) into 96 well microtitre plates with 80 µL horse serum for 24 hours at 28°C. Negative control wells were provided by using 80 µl of phosphate buffer solution (PBS) and ivermectin in PBS was used as a positive control. After 24 hours of serum exposure, the larvae and serum were transfered onto 96 well plates fitted with 20 µm filters, and larvae allowed to migrate for a further 16 hours. The larvae were then killed with iodine solution, and the number of larvae that had migrated through the 20 µm filter were counted. The numbers of larvae exhibiting normal motility, i.e. successfully migrating through the filter, were expressed as a percentage of the numbers of larvae with normal motility in control microtitre-plate wells (containing no serum). Each serum sample was tested in a minimum of two separate larval migration inhibition assays using L3s from the one faecal culture.

3.4 MHC Typing

The aim of this study was to determine whether there was any correlation between likelihood of an animal contaminating pastures with strongyle eggs (as reflected in FEC) or worm burden, and haplotype of two MHC genes, *DQA* and *DRB*.

MHC typing was performed by:

(i) DNA sequencing of the functionally important exon 2 of the class II *DQA* molecule, and
(ii) Using an indirect method based on microsatellites known to be linked to *DRB*.

DNA extraction: DNA was extracted using a salting method from samples of blood collected in heparinised tubes from 55 horses involved in the prospective study.

DQA sequencing: Previously used primers (*DQA-2e* and *DQA-2f*; Fraser and Bailey 1998) were used to amplify *DQA* exon 2 using the polymerase chain reaction (PCR). The PCR was performed with a final volume of 10 µl, containing 1X PCR buffer, 0.5 mM $MgCl_2$, 0.15 mM dNTPs, and 0.25 µM each of forward and reverse primers, 0.1 µl of Hotstar Taq (Qiagen) and approximately 10 ng of genomic DNA. The PCR products for each horse were then screened for pattern variation using single strand conformation polymorphism (SSCP). The SSCP gel consisted of 12% acrylamide:bis acrylamide (35.7:1;), 8% glycerol and 0.5 x TBE buffer (4.5 M TRIS, 4.5 M boric acid, 1 mM EDTA). The samples were denaturated by heating to 95 ^{o}C in loading dye/denaturation solution [95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF] for five minutes then kept on ice until loading. Gels were run for a minimum of 20 hours at 4^{o}C. SSCP bands were visualised by silver staining. To sequence SSCP variants, bands were removed, re-amplified by PCR and sequenced. Sequences were obtained using the BigDye Termination Kit v3.1 and capillary separation was performed on the Applied Biosystems 3130xl Genetic Analyser.

Microsatellite analysis: Microsatellites surrounding the *DRB* gene were used to indirectly screen for *DRB* variability. One published microsatellite (UM-011; Curik, Fraser et al. 2003) was genotyped. In addition, we identified four microsatellite loci from a region of the horse genome (www.ncbi.nlm.nih.gov.au/Genbank) surrounding the *DRB* gene using the program Repeatmasker (http://www.repeatmasker.org/) and the primer design program Primer3 (http://frodo.wi.mit.edu/), as detailed in Table 1.

Microsatellite loci were amplified by PCR using M13 tailing with either FAM, NED, or PET fluorescent labelling. Multiplex PCR reactions with the Qiagen Multiplex Mix were used, although some singleplex reactions were utilised (1X PCR buffer, 0.5 mM $MgCl_2$, 0.15 mM dNTPs, 0.125 µM of forward primer and 0.25 µMof reverse primer and of the appropriate M_{13} dye, 0.1 µl of Hotstar Taq (Qiagen), and approximately l0 ng of genomic DNA). PCR reactions used the following amplification profile: 15 minutes at 95^{o}C, followed by 30 cycles of 30s at 95 ^{o}C, 45s at 55 ^{o}C, and 45s at 72 ^{o}C, and eight cycles of 30s at 95 ^{o}C, 45s at 53 ^{o}C, and 45s at 72 ^{o}C, and an extension of 10 min at 72 ^{o}C. PCR

products were separated by capillary electrophoresis on the Applied Biosystems 3130xl Genetic Analyser.

Table 1: Primers for microsatellite loci identified in the region of DRB.

Name	Length	Tm*	Repeat	Sequence
KJMS1-Fp	40	59.8	$(ttta)_6$	CACAGGAAACAGCTATGACCTAGGGCAAGAGAGGGAATGA
KJMS1-R	21	60.7		TGCACACATCAGACTCAATGG
KJMS2-Ff	39	59.7	$(gt)_{17}$	TTTCCCAGTCACGACGTTGGGCACGTGGGAGTCTTTTAG
KJMS2-R	23	59.4		TTCACCCACTAAATCTCAAATCC
KJMS3-Ff	39	59.5	$(gt)_{19}$	TTTCCCAGTCACGACGTTGGAGAGCTTCACTACGCAGCA
KJMS3-R	20	60.5		GTCTCTCCACAACCCCCTCT
KJMS4-Fn	38	58.4	$(gt)_{11}$	TAAAACGACGGCCAGTGCCCCCAAAGTCATCAGTGTGT
KJMS4-R	20	60.1		GGGATGAGGACAGGCAGTAA

* Tm, theoretical melting temperature. M13 tail is underlined.

Analysis of Data: Microsatellite loci variability was assessed in PopTools (http://www.cse.csiro.au/poptools/). Phylogenetic analysis and variability of *DQA* sequences were performed in MEGA v4.0 (Tamura, Dudley et al. 2007). FEC and worm burden differences were assessed by allele and between horses grouped into heterozygotes and homozygotes for each locus. Odds ratio tests were used to assess specific alleles and heterozygosity effects among horses with high and low parasite loads.

4. Results

4.1 Prospective Study

FECs were obtained from 584 samples from 54 horses (Figure 1). A summary of the basic descriptive statistics of the dataset used in each of the analyses is presented in Table 2.

Figure 1: Histogram of 584 FECs collected over one year from 54 horses in south-east Queensland.

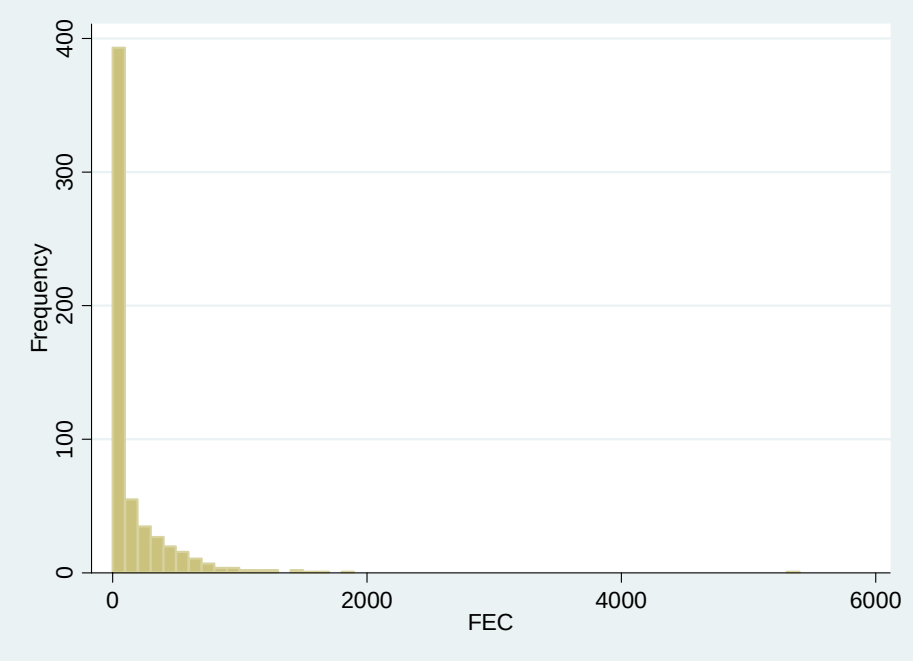


Table 2: Descriptive statistics of FEC data using the two models

Dataset	N	Mean	SD	Median	Minimum	Maximum
FEC – ZINB	584	150.8	336.0	16	0	5333
FEC - logistic	247	240.7	447.1	90	0	5333

4.1.1 Zero-inflated negative binomial analyses

The count and odds ratios for the univariable and final models are shown in Tables 3 and 4. In the logistic component of the final model, use of a macrocyclic lactone in either of the previous two months increased the likelihood of a zero count. Counts were also more likely to be zero in the summer than during any of the other seasons, and less likely to be zero in ponies and Arab/ArabX animals compared to the other breeds. In the negative binomial component of the final model, counts

were likely to be lower in horses treated two months previously with a macrocyclic lactone, higher in ponies compared to other breeds, and higher in stallions compared to mares or geldings.

Table 3: Count ratios and odds ratios for univariable zero-inflated negative binomial models for faecal egg count, using 584 samples from 54 horses on nine south-east Queensland properties.

Variable				**p-value**
Negative binomial component	No. horses	No FECs	**Count Ratio**[1] **(CI)**	
Anthelmintic previous month				
None	N/A	434	Reference	
Benzimidazole	N/A	40	1.269 (0.925, 1.744)	0.14
Macrocyclic lactone	N/A	110	0.615 (0.122, 3.103)	0.556
Anthelmintic 2 mo previous				
None	N/A	446	Reference	
Benzimidazole	N/A	40	1.278 (0.953, 1.712)	0.102
Macrocyclic lactone	N/A	98	0 .484 (0.308, 0.760)	0.002
Age				
<=5	12	142	Reference	
>5	42	442	1.072 (0.704, 1.632)	0.746
Breed				
Quarter horse/Quarter horse x/stockhorse/thoroughbred	26	290	Reference	
Warmblood	11	100	1.012 (0.576, 1.777)	0.967
Pony	3	31	1.933 (0.586, 6.381)	0.279
Arab/Arab x	14	163	0.832 (0.586, 1.182)	0.305
Sex				
Mare/gelding	48	532	Reference	
Stallion	6	52	1.84 (1.115, 3.038)	0.017
Property				
1	8	96	Reference	
2	6	70	0.226 (0.081, 0.633)	0.005
3	8	64	0.659 (0.285, 1.524)	0.33
4	5	60	0.760 (0.358, 1.617)	0.477
5	4	47	0.673 (0.339, 1.335)	0.257

6	3	36	0.200 (0.102, 0.392)	<0.001
7	3	35	0.312 (0.133, 0.734)	0.008
8	11	128	0.538 (0.275, 1.052)	0.07
9	6	48	0.673 (0.259, 1.750)	0.417
Period				
Summer	N/A	147	Reference	
Autumn	N/A	159	0.980 (0.643, 1.4940	0.926
Winter	N/A	162	0.818 (0.543, 1.235)	0.34
Spring	N/A	116	1.159 (0.595, 2.257)	0.578

Logistic component			**Odds Ratio[2] (CI)**	
Anthelmintic previous month				
None	N/A	434	Reference	
Benzimidazole	N/A	40	0.799 (0.105, 1.638)	0.557
Macrocyclic lactone	N/A	110	60.000 (18.228, 197.157)	<0.001
Anthelmintic 2 mo previous				
None	N/A	446	Reference	
Benzimidazole	N/A	40	0.796 (0.442, 1.434)	0.448
Macrocyclic lactone	N/A	98	2.263 (1.210, 4.235)	0.011
Age				
<=5	12	142	Reference	
>5	42	442	0.793 (0.426, 1.476)	0.464
Breed				
Quarter horse/Quarter horse x/stockhorse/thoroughbred	26	290	Reference	
Warmblood	11	100	1.155 (0.520, 2.570)	0.723
Pony	3	31	0.352 (0.224, 0.555)	<0.001
Arab/Arab x	14	163	0.532(0.325, 0.870)	0.012
Sex				
Mare/gelding	48	532	Reference	
Stallion	6	52	0.582 (0.254, 1.334)	0.201
Property				
1	8	96	Reference	

2	6	70	1.898 (0.522, 6.896)	0.331
3	8	64	0.810 (0.308, 2.135)	0.67
4	5	60	1.509 (0.592, 3.846)	0.388
5	4	47	0.776 (0.399, 1.508)	0.455
6	3	36	0.402 (0.202, 0.802)	0.01
7	3	35	0.420 (0.203, 0.869)	0.019
8	11	128	0.517 (0.264, 1.016)	0.056
9	6	48	0.298 (0.066, 1.349)	0.116
Season				
Summer	N/A	147	Reference	
Autumn	N/A	159	0.248 (0.134, 0.459)	<0.001
Winter	N/A	162	0.480 (0.288, 0.799)	0.005
Spring	N/A	116	0.548 (0.311, 0.966)	0.038

[1] Estimated ratio of mean faecal egg count for horses in the exposed group relative to the reference group

[2]Odds ratio for horses in the exposed group having a zero count relative to the reference group

Table 4: Count ratios and odds ratios for the final, multi-variable zero-inflated negative binomial model for faecal egg count, using 584 samples from 54 horses on nine south-east Queensland properties.

Variable		
Negative binominal component	**Count Ratio[1] (CI)**	**p-value**
Anthelmintic 2 mo previous		
None	Reference	
Benzimidazole	1.35 (0.99, 1.84)	0.061
Macrocyclic lactone	0.54 (0.37, 0.78)	0.001
Breed		
Quarter horse/quarter horse x/stockhorse /thoroughbred	Reference	
Warmblood	0.79 (0.52, 1.18)	0.251
Pony	2.14 (0.66, 6.94)	0.204
Arab/arab x	1.02 (0.72, 1.43)	0.925
Sex		
Mare/gelding	Reference	
Stallion	2.24 (1.50, 3.36)	<0.001

Logistic Component	**Odds Ratio[2] (CI)**	**p-value**
Variable		
Anthelmintic previous month		
None	Reference	
Benzimidazole	1.02 (0.44, 2.36)	0.959
Macrocyclic lactone	181.82 (39.21, 843.03)	<0.001
Breed		
Quarter horse/quarter horse x/stockhorse/thoroughbred	Reference	
Warmblood	0.97 (0.27, 3.47)	0.956
Pony	0.24 (0.10, 0.59)	0.002
Arab/arab x	0.15 (0.07, 0.35)	<0.001
Anthelmintic 2 mo previous		
None	Reference	
BZ	0.58 (0.23, 1.48)	0.256
ML	7.66 (4.02, 14.61)	<0.001
Season		
Summer	Reference	
Autumn	0.23 (0.11, 0.47)	<0.001
Winter	0.31 (0.15, 0.64)	0.002
Spring	0.33 (0.15, 0.75)	0.008

[1] Estimated ratio of mean faecal egg count for horses in the exposed group relative to the reference group

[2]Odds ratio for horses in the exposed group having a zero count relative to the reference group

4.1.2 Logistic regression for estimation of ICC

Once FEC data from horses that had received macrocyclic lactone treatment in the previous three months were excluded, the reduced dataset comprised 247 FECs from 53 horses with 2 – 11 (mean 4.7) FECs per horse (Figure 2, Table 5).

Figure 2: Histogram of 247 FECs collected over 12 months from 53 horses that had not been treated with a macrocyclic lactone in the previous three months.

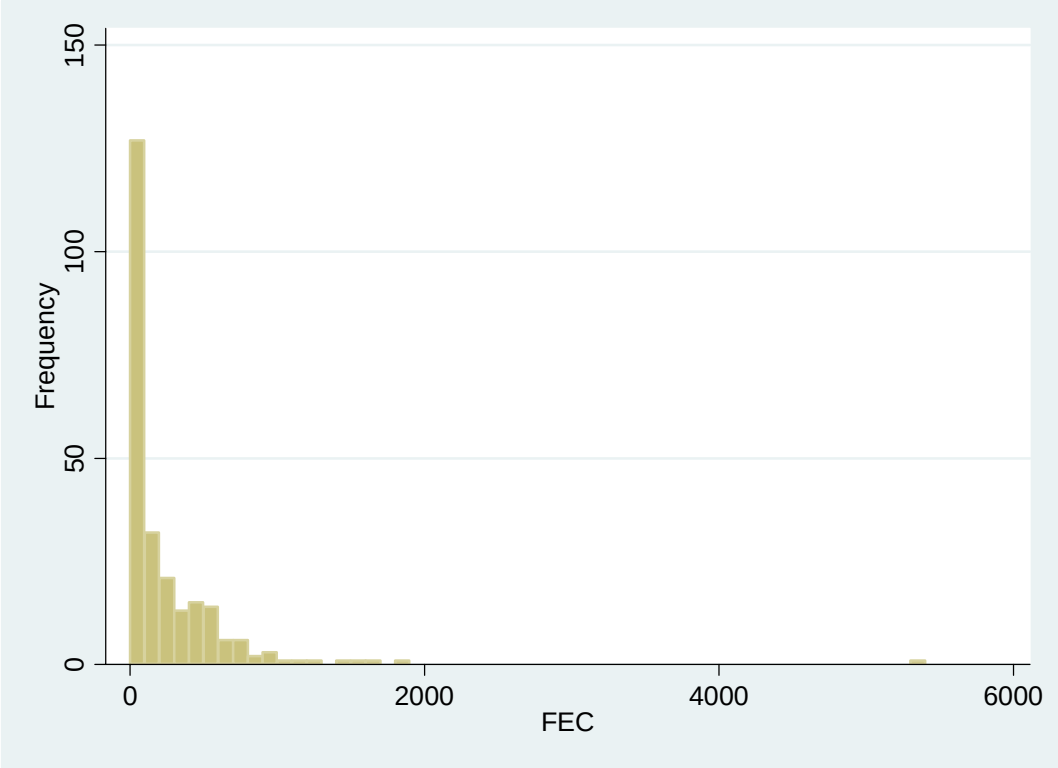


Table 5: Number of horses and FECs for each explanatory variable for random effects logistic models

Variable	No. horses	No FECs
Age		
<=5	12	52
>5	41	195
Breed		
Quarter horse/quarter horse x/stockhorse/thoroughbred	26	153
Warmblood	11	40
Pony	3	19
Arab/arab x	13	35
Sex		
Mare/gelding	47	224
Stallion	6	23
Property		
1	8	40
2	6	46
3	8	22
4	5	15
5	4	18
6	3	29
7	3	15
8	10	20
9	6	42
Period		
Summer	N/A	29
Autumn	N/A	85
Winter	N/A	84
Spring	N/A	49

Table 6: Estimates of the within-horse correlation in FECs from random effects logistic regression models.

Model	**Null**		**Property as fixed effect**	
Outcome	*ICC (95% CI)*	*p-value*	*ICC (95% CI)*	*p-value*
0/>0	0.804 (0.624, 0.910)	<0.0001	0.751 (0.537, 0.886)	<0.0001
<50/>=50	0.764 (0.571, 0.887)	<0.0001	0.701 (0.486, 0.853)	<0.0001
<100/>=100	0.721 (0.533, 0.854)	<0.0001	0.627 (0.415, 0.800)	<0.0001
<200/>=200	0.655 (0.464, 0.807)	<0.0001	0.505 (0.298, 0.710)	<0.0001
<500/>=500	0.578 (0.346, 0.781)	<0.0001	0.400 (0.177, 0.674)	<0.0001

Figure 3: Histogram of the percentage of FECs ≥100epg for individual horses using data from 247 FECs from 53 horses collected over one year.

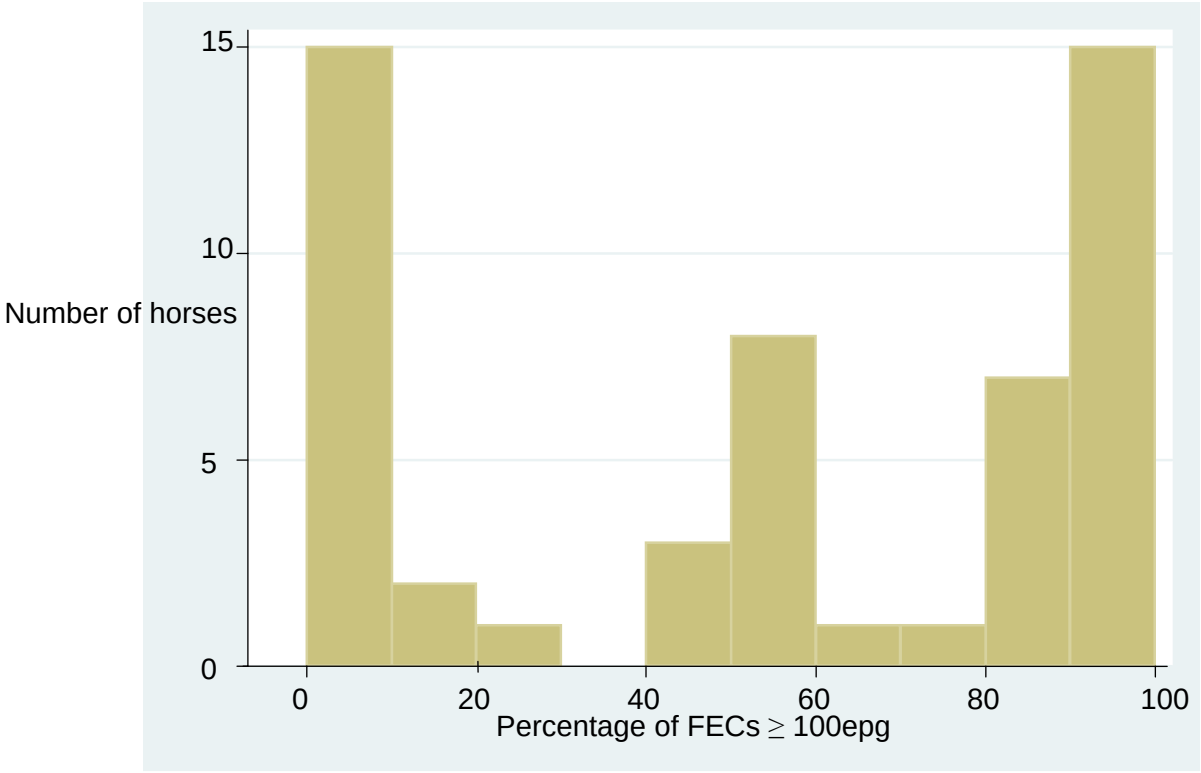
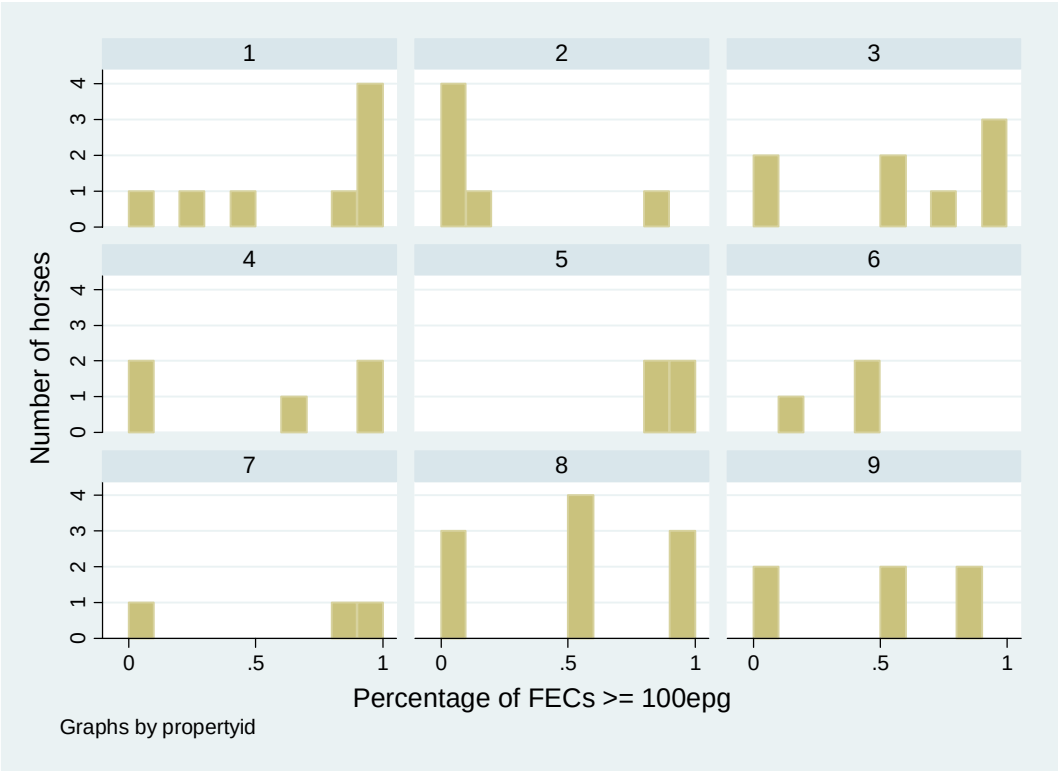


Figure 4: Histogram of the percentage of FECs ≥100epg for individual horses by property (1-9) using data from 247 FECs from 53 horses collected over one year.



The intraclass correlation co-efficients are high for each of the selected outcome variables indicating FECs within horses were strongly correlated across a wide range of FECs over the sampling period (Table 6). There is very little change in the ICC when age, breed, sex or season are fitted to the model indicating that these putative explanatory variables do not explain much of the clustering in the data. A small proportion of the clustering may be explained at the property level as fitting property as a fixed effect reduces the ICC by ~ 7 – 30% (Table 6).

The distribution of the percentage of individual horse's counts greater than or equal to 100 epg is multimodal with the highest peaks in the 0 – 10% and 90 – 100% categories (Fig 3). This is highly supportive of clustering of FECs within horses. The distributions are also widely spread and commonly bimodal when examined at the property-level (Fig 4), which provides further support that the clustering reflects horse-level rather than property-level factors.

The conclusions from the prospective study were that:

1) Outcomes from a study of this type have the potential for bias resulting from selective removal of data. For example, those horses on properties where the mean FEC rose above 200epg on a more frequent basis were under-represented, as such animals were treated more frequently with anthelmintics and not included in the logistic regression for estimation of ICC. Thus the dataset is biased towards horses that consistently contribute little to pasture contamination and/or graze paddocks which are not severely contaminated by other high-shedding horses. For welfare reasons, and in the interests of our participating properties, this was unavoidable.

2) Despite this reservation, the ICC demonstrated a clustering of FEC within horses or, in other words, this data supported the hypothesis that a small number of individual horses contributed disproportionately fewer eggs to contaminate pastures with cyathostomins. Because of the bias alluded to in Point 1, it is not possible to assert the converse (i.e. that a small number of horses contribute disproportionately more to pasture contamination).

3) Clustering of FEC at the property level appears to be less important than individual horse factors. This suggests that variables such as pasture management strategies, behaviour and grazing pressure in this trial were not significantly contributing to FEC levels. However paddock-level effects cannot be entirely dismissed as we did not collect details of pasture management and animal husbandry (e.g. which horses were in which paddocks, when and with what other animals).

4) Treatment with a macrocyclic lactone in the two months prior to sampling was associated with a greater likelihood of a zero FEC, but treatment with a benzimidazole product in this time was not.

5) Stallions were likely to have higher FEC than geldings or mares.

6) Ponies were likely to have higher FEC than the other breed groups examined.

4.2 Abattoir Survey

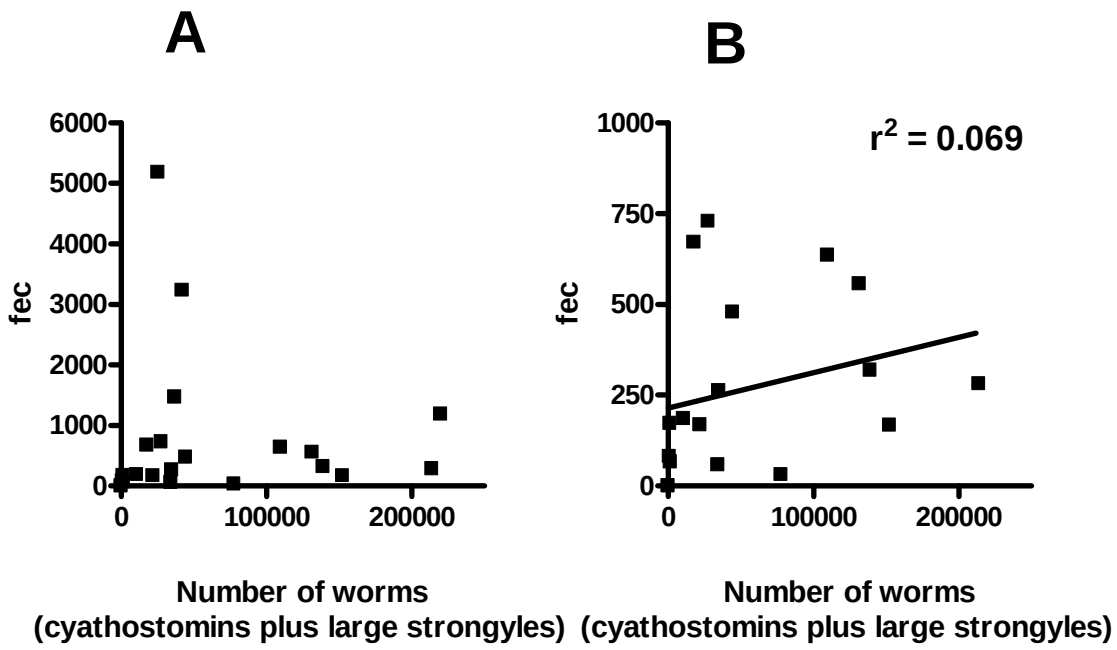
Worm counts were completed for 22 horses. The data is shown in Table 7 below. The worms were predominantly luminal cyathostomins, with a very small percentage of large strongyles also recovered from some horses.

Table 7: Faecal egg count (FEC) and total luminal worm count at necropsy of 22 horses.

Horse number	Total number of worms	% large strongyles (remainder were luminal cyathostomins)	FEC (eggs per gram)
1	17880	0	671
2	1014	0	81
3	34971	0.0086	262
4	44410	0.027	479
5	219901	0.025	1189
6	77881	0	31
7	131497	0.0076	556
8	27549	0	729
9	60	0	0
10	22003	0	168
11	213825	0	281
12	111	0	0
13	10681	0.075	185
14	1326	0	172
15	34286	0	58
16	152344	0.0098	167
17	139026	0.030	319
18	36965	0	1468
19	109744	0.074	635
20	1476	0	66
21	25442	0.0040	5183
22	42217	0	3233

The relationship between FEC and total numbers of worms (both cyathostomins and large strongyles) is shown in Fig 5; there was no correlation between the two variables. The three highest FEC data points were from horses with significantly fewer worms than other horses examined in the study. We therefore also looked at whether omission of these highest FEC data points might permit the FEC to be a useful predictor of worm burden (Figure 5B), however, it was again apparent that there was no correlation between the two variables, even when four counts over 1000 epg were omitted.

Figure 5. Faecal egg count (FEC) vs. total number of worms (luminal cyathostomins and large strongyles) in 22 horses; A, all data; B, only cases with FEC < 1,000.

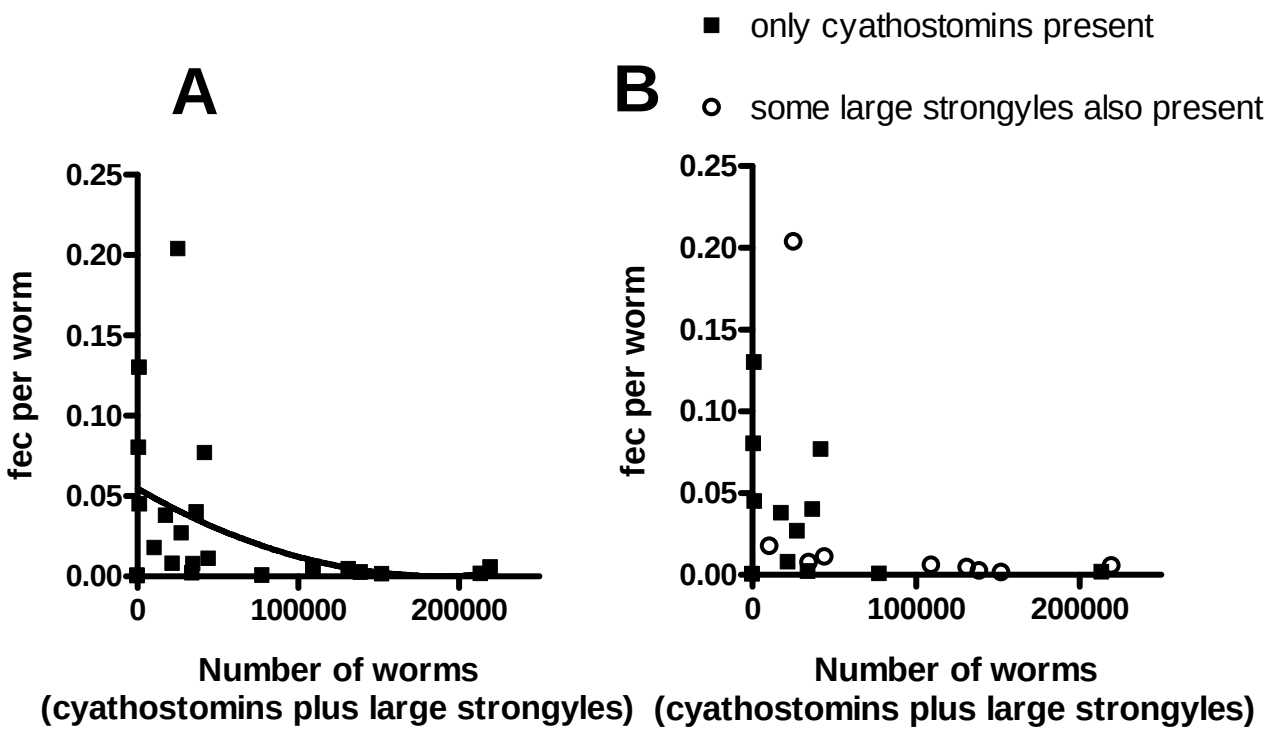


We were interested in whether the observed lack of correlation between FEC and worm burden may have been due to density-dependent worm fecundity effects. This phenomenon has been reported in other helminth species. For the present study we had no data on faecal output of each horse and hence could not directly examine the total output of worm eggs per horse over a defined period (e.g. 24 hours) in order to accurately calculate egg output per worm. Hence, as an approximation of output per worm we looked at the contribution made by each worm in a particular horse to the total FEC for that animal by dividing the FEC by the number of worms recovered. The data is shown in Fig 6.

If density-dependent fecundity effects were absent, the graph would show a horizontal line, indicating that worms contribute in a uniform and unchanging manner to total FEC as the worm number increases. It is clear from Fig 6A, however, that this is not the case. A strong density dependence is apparent, with a reduction in FEC per worm as the worm density increases. Those horses with a small proportion of large strongyles did not form a distinct cluster separate from the horses possessing only cyathostomins (Fig 6B). Hence it is also clear that the presence of a small proportion of large strongyles in some horses is not responsible for the observed density-dependent fecundity effects.

Figure 6: Relationship between worm number and FEC on a per worm basis.

The data indicate the contribution of each worm in a particular horse to the total FEC for that animal. **A**, all data; **B,** data shown as animals in which only cyathostomins were recovered, and those in which a small percentage of large strongyles were also present.



The two features common to helminth density-dependent fecundity relationships are apparent:

1) decreased fecundity at higher worm burdens (due to density-dependent constraints, most likely related to host immune response), and
2) a greater degree of variability at low worm densities (due presumably to variation between individual horses in terms of their ability to mount an effective immune response to worms present in relatively low numbers).

The potential significance of density-dependent worm fecundity, in terms of managing worm infections in horses, is two-fold:

1) it may be responsible for the lack of correlation between FEC and worm burden observed in Figure 5, and/or
2) it has the potential to impact significantly in the use of the FECRT for monitoring drug resistance. Kotze and Kopp (2008) recently showed that density dependency is a dynamic phenomenon with the canine hookworm in relation to anthelmintic exposure. They showed that the hookworms present after a drug treatment are able to greatly increase their egg output, presumably due to the relaxation of density-dependent constraints after the treatment. This leads to an underestimation of drug efficacy based on a comparison of pre- and post-drug FECs (as used in Faecal Egg Count Reduction Tests, FECRTs).

The conclusions from the abattoirs were that:

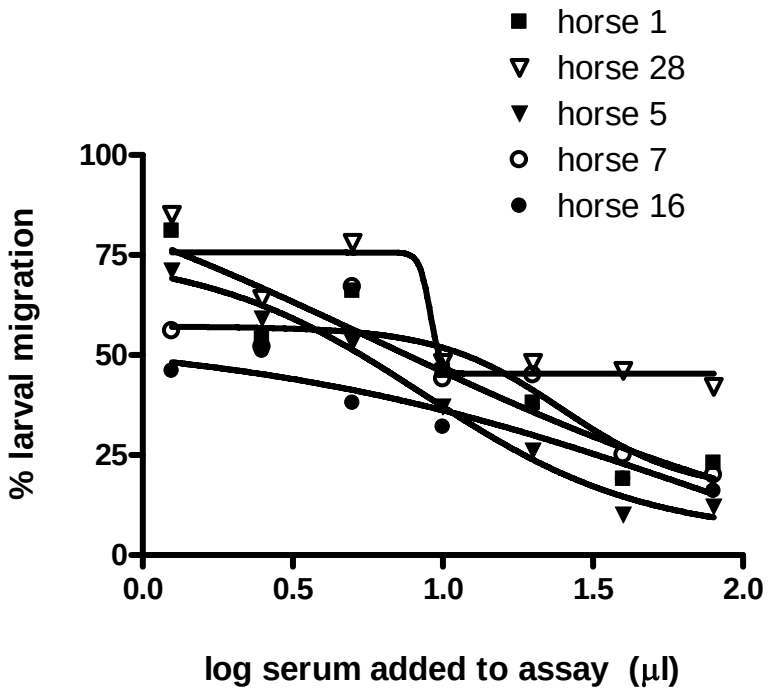
1) FEC was not correlated to worm burden in the horses examined. This indicated that FEC is a poor method for monitoring infection levels. On the other hand, the FEC will indicate the relative degree of pasture contamination likely to occur with individual horses.

2) There is strong density dependency in worm fecundity for horse cyathostomins. This may explain the poor correlation between FEC and worm burden relationship, and has implications for the use of the FECRT for measuring anthelmintic resistance levels.

4.3 Larval Migration Inhibition Activity

Horse serum can inhibit the movement of cyathostomin larvae in *in vitro* migration assays (Figure 7). In the assays shown here, the degree of inhibition of migration increased as the concentration of serum in the assays was increased, indicating a dose responsiveness for the assay. The nature of the dose response varied among the different sera, with some showing a lower slope, and one sample in particular showing the presence of a distinct plateau at a migration level of approximately 50 %.

Figure 7. Dose response of cyathostomin larvae in migration assays with different concentrations of serum from five horses.



We were interested in whether the effect of serum on larval migration was correlated with the FEC data we had collected over time from each horse in our prospective study. A significant correlation would suggest that the inhibitory activity possessed by the serum was related to the ability of the horse to resist infection by worms and/or shed worm eggs onto pastures.

We examined the serum migration data in several ways:

1) with respect to the mean FEC for each horse over the course of the study as a measure of overall resistance to infection (Figure 8),

2) with respect to the FEC at the time that the serum was taken (the beginning of the study) as a measure of the horse's contribution to pasture contamination at the time of serum sampling (Figure 9), and

3) with respect to the trend in FEC for each horse over the four weeks following serum collection, as a measure of whether the horse was mounting an effective immune response (actively expelling worms) during the period immediately following serum collection (Figure 10). This analysis could only be performed on a subset of the data as some properties drenched their horses several days after the serum sampling.

Figure 8. Relationship between effect of serum (sampled at the beginning of the study) on larval migration and the mean FEC for each horse over the length of the study.

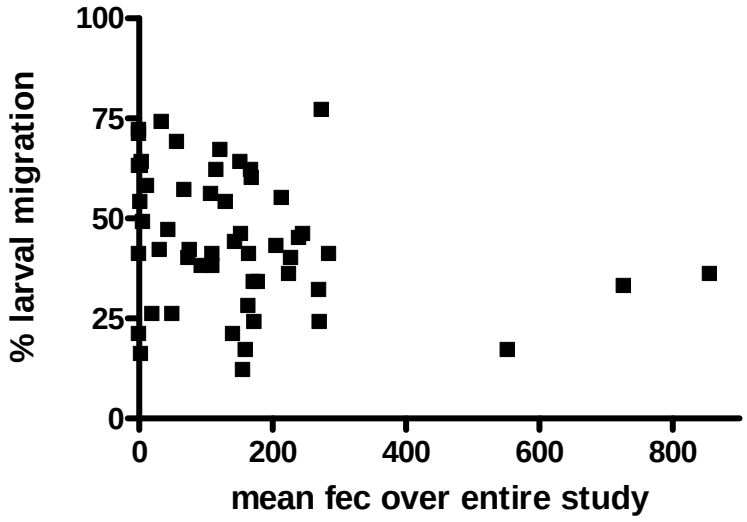


Figure 9. Relationship between effect of serum on larval migration and the FEC of each horse on the day that serum was sampled.

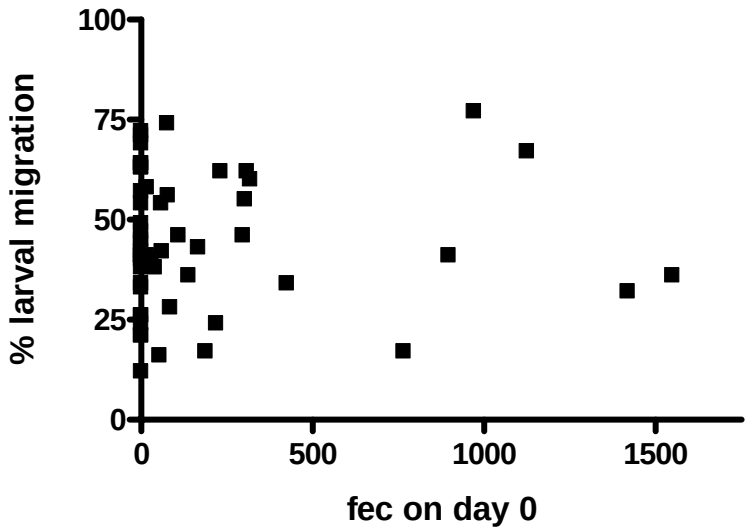
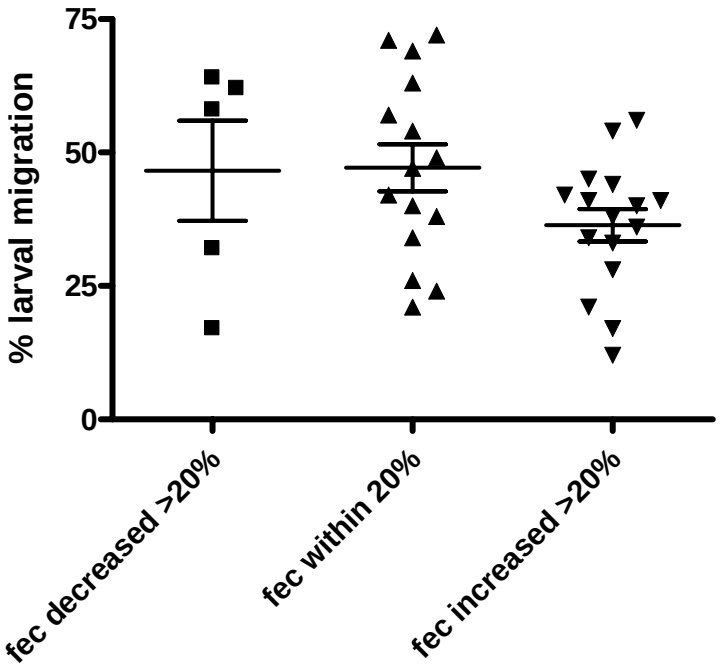


Figure 10. Relationship between effect of serum on larval migration and the change in FEC occurring between the day of serum sampling and a time point 28 days later.

The mean ± SE is shown for each data set. There was no significant difference between the means at *P* = 0.05.



It is clear from these graphs that there was no significant correlation between the larval migration inhibition activity observed in horse serum and FEC. Hence, the serum assay cannot be used as a predictor of a horse's likely level of contamination of pastures. Because of the poor correlation between FEC and worm burden, it is not yet clear whether there is any correlation between larval migration inhibition activity and a horse's ability to withstand infection.

The migration inhibitory effects of serum are poorly understood. Little work has been reported in the scientific literature to date on the nature of the inhibitory factors, and how they relate to the overall immune status of an animal. Claerebout et al (1999) showed that serum from *Ostertagia*-infected cattle inhibited larval motility and migration. Serum larval motility inhibition (LMI) was significantly negatively correlated with *Ostertagia* worm counts. Giacomin, et al (2005) have shown that similar larval inhibitory effects occur in a worm/mouse model, and have implicated complement in the observed anthelmintic effects. Similarly, Kotze (unpublished data) has observed that complement in serum from sheep can strongly inhibit migration of *Haemonchus contortus* larvae *in vitro*. The most recent sheep data (Kotze, unpublished) suggests that the phenomenon is strongest in helminth-naïve sheep, and hence, if it is associated directly with *in vivo* response to worms, it may be predominantly an innate immunity mechanism. That is, it may operate in young animals as an early immune response, distinct from later acquired immunity after repeated exposure to parasites. The same system may be operating in horses. It appears likely from the present study that the serum effects were not related to immunity of the horses examined here. Given that the most recent indications from sheep are that the migration inhibition factors seem to be more associated with innate immunity, it may be worthwhile examining whether the effects are more significant in serum from young horses. It remains possible therefore that the serum assay may be an indictor of innate immune status in these animals.

The conclusions from the larval migration inhibition study were that:

1) Serum from some horses possesses an agent with anthelmintic activity as measured in *in vitro* larval migration assays.

2) The anthelmintic activity present in the serum does not correlate with an animal's level of pasture contamination with L3 stage larvae.

3) It appears unlikely that the anthelmintic activity present in the serum is correlated with ability to withstand worm infections, or worm burdens, but further work is required to confirm this.

4) These observations were limited to horses over two years of age, and further work with younger horses is warranted.

4.4 MHC typing

A total of 55 horses were typed for DRB-linked microsatellite loci and DQA alleles. Substantial variation was identified for both loci. The microsatellite loci had an average number of alleles per locus of 9.5 (sd 2.89), with a range of 7 to 12 alleles (Table 8). Overall observed heterozygosity was 59.1% and unbiased expected heterozygosity was 75.8%. The heterozygosity of individual horses varied from 0 (homozygous at all four loci) to 1 (heterozygous at all four loci), with an average of 0.6. Missing data is present at 3.2%.

4.4.1 DRB analysis

Table 8: Microsatellite variability in tested horses from the prospective study.

Locus	Number of alleles	Observed heterozygosity	Expected heterozygosity	PIC*
KJMS2	7	0.67	0.78	0.75
KJMS3	12	0.42	0.73	0.71
KJMS4	7	0.44	0.71	0.65
UM011	12	0.84	0.81	0.78

* PIC; polymorphism information content

MHC typing is reported for the horses from the prospective trial so any associations relate to the extent of pasture contamination with worm eggs by an individual horse (as opposed to total cyathostomin burden).

Parasite resistance or susceptibility has previously been associated with both heterozygosity level and with individual alleles. For the microsatellite loci, there was only a weak negative association between heterozygosity across all loci and FEC (Figure 11). Although homozygotes at three of the four loci (Fig 12) tend to have higher mean FEC values, there was substantial intra-group variation. However, individual resistance allele effects were more apparent. There were four alleles associated with low mean FEC, that is, that show mean FEC values of less than 100 calculated over at least five horses. These alleles were KJMS2-293, KJMS3-215, KJMS4-259 and UMO11-192 (Table 9). Categorising horses as ‘low’ or ‘high’ mean FEC (i.e. as less or more than 200 epg), there were no horses with ‘high’ values that carried alleles KJMS2-293, KJMS3-215 or UMO11-192. There were no susceptibility alleles identified that were consistently associated with high FEC.

Figure 11: Association between FEC and heterozygosity at DRB-linked microsatellite loci.

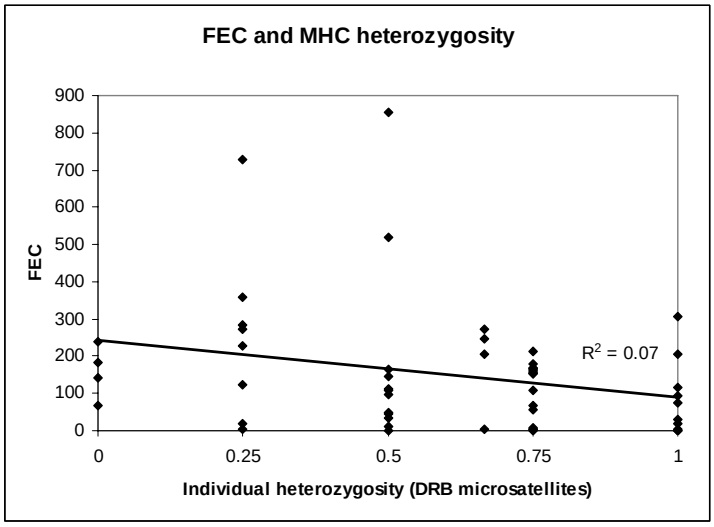
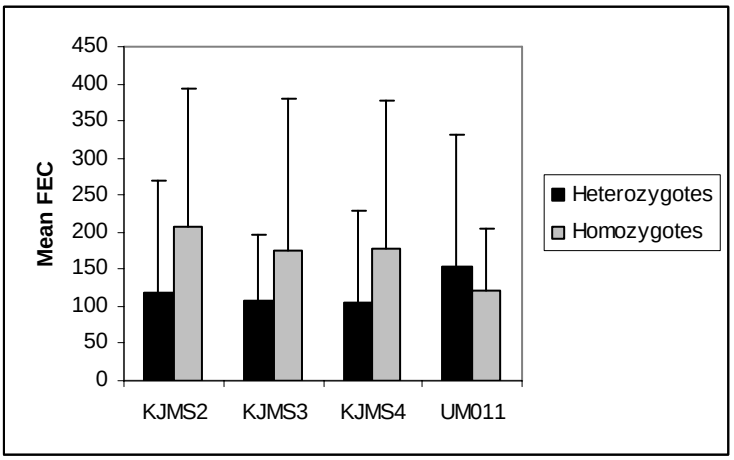


Figure 12: Mean FEC for DRB-linked microsatellite alleles for heterozygous and homozygous horses.



(Y error bars depict standard deviation)

Table 9: FEC for prospective trial horses by DRB-linked microsatellite allele.

Locus	Allele	Mean FEC	Minimum FEC	Maximum FEC	Number of individuals with allele
KJMS 2	293	36.5	0.0	160.5	5
	308	129.4	33.6	213.8	8
	310	112.4	0.0	273.8	23
	312	129.7	0.0	273.8	7
	316	109.0	2.7	359.2	8
	319	168.1	0.0	856.2	28
	321	170.2	0.0	856.2	13
KJMS 3	213	31.5	31.5	31.5	1
	215	39.5	0.0	160.5	6
	217	141.4	31.5	306.4	5
	221	223.2	0.0	727.3	8
	224	145.5	33.6	284.9	7
	228	108.7	2.8	213.8	3
	232	113.3	0.0	306.4	33
	236	250.7	94.1	856.2	7
	240	182.2	5.1	359.2	2
	242	17.0	17.0	17.0	1
	315	19.7	19.7	19.7	1
	323	205.2	205.2	205.2	1
KJMS 4	232	306.4	306.4	306.4	1
	252	117.8	0.0	520.3	20
	257	174.9	0.0	727.3	16
	259	99.2	0.0	271.4	28
	261	220.2	31.5	856.2	5
	296	152.2	152.2	152.2	1
	310	152.2	152.2	152.2	1
UMO 11	179	168.2	94.1	271.4	6
	182	171.7	0.0	856.2	17
	183	109.9	109.7	110.2	2
	184	129.0	33.6	213.8	4
	185	136.2	0.0	856.2	34
	187	118.0	0.0	520.3	8
	188	359.2	359.2	359.2	1
	189	213.2	0.0	727.3	12
	190	246.5	246.5	246.5	1
	191	138.5	57.0	246.5	3
	192	33.2	0.0	160.5	6
	197	165.3	31.5	306.4	7

4.4.2 DQA analysis

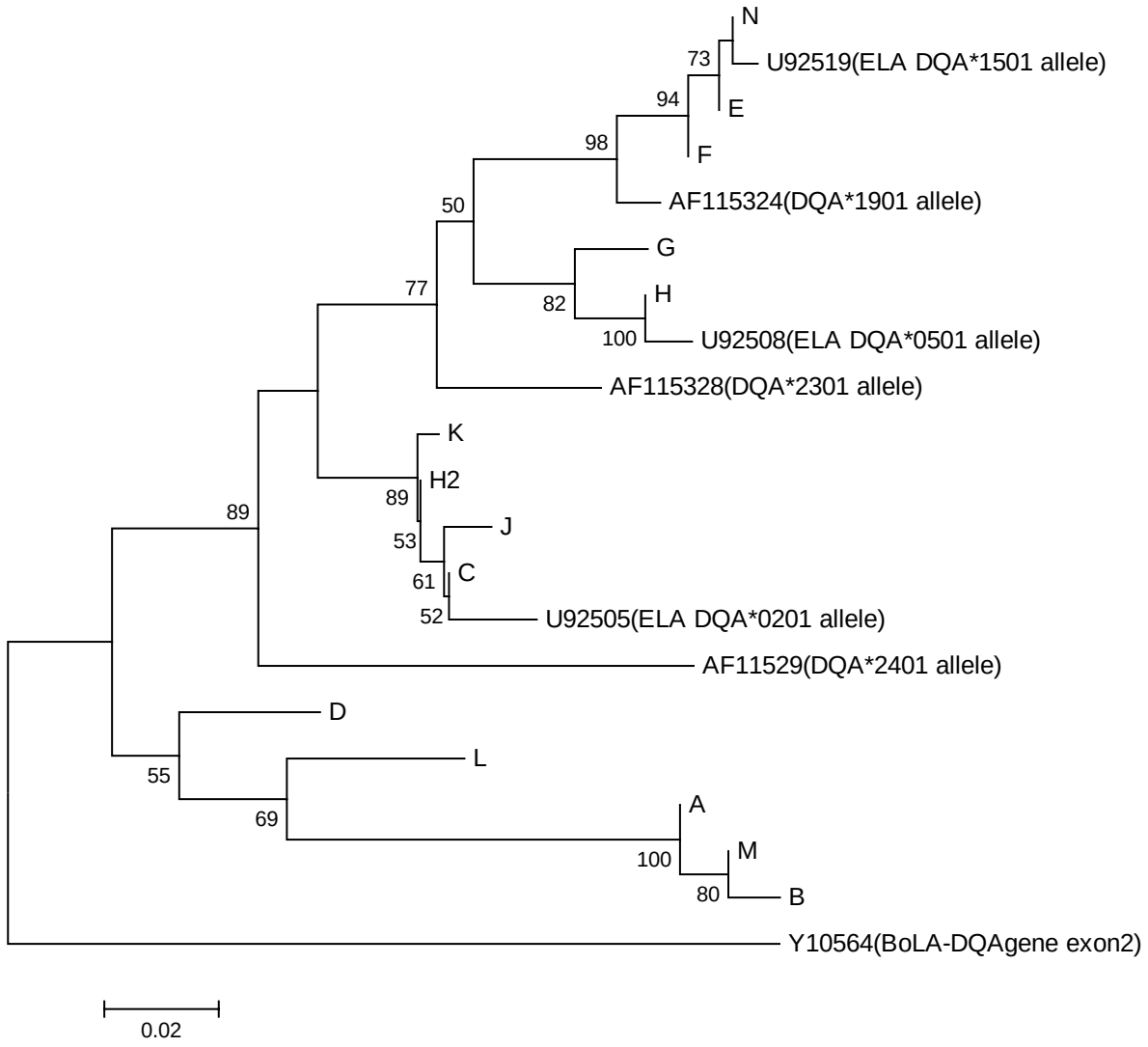
DQA typing by SSCP was difficult and indistinct. However, one to two alleles could be assigned for each typed horse from the prospective study and DNA sequences represented in each band type were confirmed by DNA sequencing of gel cuts. A total of 14 alleles were identified, seven of which had been previously published (Table 10). One allele (A), which was identical to ELA-DQA*1301, was common. Previous research has also found this allele to be common among tested horses, and suggested it related to a locus separate from the MHC region on chromosome 20 (Fraser and Bailey 1998). Hence the alleles presented here may not relate to a single locus. The relationship among alleles is shown in Figure 13; it does not give any indication that alleles originate from two separate loci.

Table 10: DQA alleles from prospective trial horses and comparison with previously published alleles.

Project allele identification	Number of bands sequenced	Frequency	Published Alleles
A	5	0.55	ELA-DQA*1301
B	1	0.01	
C	2	0.19	ELA-DQA*0801
D	3	0.03	
E	2	0.03	ELA-DQA*1501
F	1	0.02	ELA-DQA*0601
G	1	0.03	ELA-DQA*0401
H	1	0.06	
H2	1	0.01	ELA-DQA*0301
J	1	0.02	ELA-DQA*0901
K	1	0.02	
L	1	0.01	
M	1	0.02	
N	1	0.01	

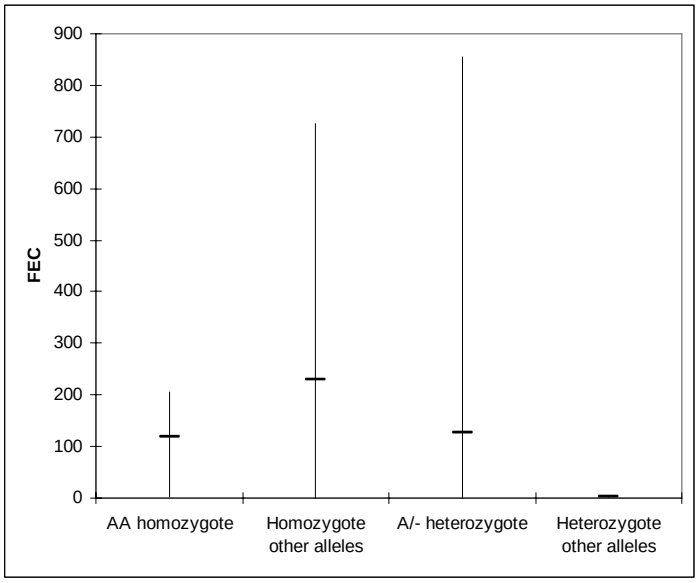
Figure 13: Phylogenetic tree of DQA alleles identified among prospective trial horses.

A selection of previously published alleles are included for comparison. The tree is rooted with a DQA sequence from cattle. The figure presents a neighbour joining tree based on Kimura two parameter distance, with bootstrap values from 1000 iterations shown where values exceed 50%.



There was no significant difference between mean FEC for heterozygotes and homozygotes at DQA (heterozygotes 147, homozygotes 149, p=0.968, OR 0.89 CI 0.22-3.53). Individual horses that were AA homozygotes showed lower average FEC than those homozygous for other alleles (Figure 14), although this difference was not significant (mean FEC for AA homozygotes 112, other homozygotes 231; p=0.419). The only other allele in high frequency was the C allele and there was no significant difference in mean FEC associated with the presence of this allele (mean FEC horses bearing C allele 182, horses without C allele 127; p=0.315).

Figure 14: Relationship between mean FEC and DQA allele categories.



(Vertical lines show minimum and maximum values)

The conclusions from the MHC typing study were that:

1) There are some DRB-linked microsatellite alleles that seem to be associated with low FEC and hence with low contamination of pastures. Further analysis on larger sample sizes is clearly warranted.

2) There is no association between heterozygosity or specific alleles at the DQA locus and the mean observed FEC in this dataset.

5. Implications

This study examined potential tools to identify horses that contribute disproportionately to pasture contamination with cyathostomin larvae. However it was not able to identify a tool to easily identify horses with lower (or higher) worm burdens *per se*. Further work on the immune response of horses to cyathostomin infections is warranted. Much of this work was focused on the diagnosis and pathophysiology of the disease larval cyathostominosis, but there is also a need for more broadly-based work looking at variations in the way animals respond to cyathostomin infections. Such work has the potential to enable the development of more strategic approaches to worm control in the horse industry, thus leading to improved economic returns for properties and prolonged useful life for the ML drugs and any new anthelmintic drug classes in the future.

This pilot project explored the feasibility of developing a marker that would allow an individual horse's cyathostomin resistance status, or propensity to contribute disproportionately to pasture contamination with cyathostomins, to be determined. In order for such a test to be useful to the horse industry, it would ideally be an assay that would need to be performed once only on an individual animal. In this respect a genetic marker approach would offer considerable advantages.

Correlations of faecal egg counts within horses over time suggested that there are important inherited and/or acquired factors that vary between horses and affect the likelihood of an individual contributing to pasture contamination with parasites (as assessed by faecal egg count) over prolonged periods. While this may not be directly related to worm burdens, it is still of relevance in developing worm control programs as it provides an avenue for targeting pasture contamination in worm control strategies.

The larval migration inhibition assay revealed that a horse's serum is able to inhibit cyathostomin larval motility. Unfortunately, in this pilot survey there was little evidence of correlation between the intensity of this inhibitory activity and luminal strongyle burdens or FEC. However further work on this assay with larger numbers of horses, and including younger animals, is required before this assay is eliminated from consideration.

MHC typing offered most promise. Four DRB-linked microsatellite alleles were associated with low mean FEC (i.e. mean FEC values of less than 100). Nevertheless, no association could be demonstrated between the DAQ locus and FEC in the samples examined. Further analysis of DRB with larger numbers of horses, and of horses with known worm burdens, currently offers the best hope of a simple test to identify whether a horse is likely to consistently contribute disproportionately to pasture contamination.

An unexpected finding in this study was the evidence that cyathostomin fecundity was influenced by worm density. The potential significance of this for the industry is two-fold:

- it complicates the interpretation of results from the Faecal Egg Count Reduction Test (FECRT), the standard test for anthelmintic resistance in the horse. Worms that survive a drug treatment are likely to significantly increase their egg output. This may lead to an underestimation of drug efficacy based on a comparison of pre- and post- drug FECs.
- it may facilitate the development of anthelmintic resistance, because following treatment there is a boost in the fecundity of worms that have survived the treatment (i.e. those with some degree of resistance to the drug).

6. Recommendations

1. This study identified potential tools to identify horses contributing disproportionately to pasture contamination with cyathostomin larvae. However it was not able to identify a tool to easily identify horses with lower (or higher) worm burdens, or resistance, *per se*. Further work on the immune response of horses to cyathostomin infections is warranted, as it will likely facilitate the development of more strategic approaches to worm control in the horse.

2. Further investigations of the apparent population density-dependent effects on cyathostomin fecundity are clearly warranted, as this phenomenon may impact both on the development of drug resistance and its detection (via the FECRT).

3. Further analysis of the relationship between DRB alleles and FEC are warranted, as this gene is the most promising candidate for a simple test to determine whether an individual horse is likely to consistently shed fewer or more worm eggs onto pastures than other animals in its group.

4. Further evaluation of the use of a horse's serum in a cyathostomin larval migration inhibition assay is also warranted.

5. When monitoring levels of pasture contamination, horse owners should be aware that stallions and pony breeds are likely to have higher FEC than geldings/mares or other breeds.

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Identification of Horses with Resistance to Small Strongyles

RIRDC Publication No. 09/081

By Glen Coleman, Jennifer Seddon, & Kim Jell

Over the last twenty years, small strongyles or cyathostomins have emerged as the most important internal parasites of the horse. The significance of cyathostomins to the horse industry lies in both their pathogenicity (as agents of colic and/or clinical larval cyathostominosis) and in their resistance to anthelmintics.

The project investigates the feasibility of developing a simple test (or tests) for the identification of an individual horse's resistance to cyathostomin infections and/or contribution to pasture contamination with nematodes. Such a test would facilitate the development of more rational nematode control strategies by identifying those animals that may usefully be targeted for more (or less) regular anthelmintic treatments.

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