Facing Disease Challenges through Novel Approaches in Molecular biology, Epidemiology and Cell biochemistry

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Abstract

Project 1: A step towards characterising a microRNA regulatory system in the parasitic nematode *Haemonchus contortus*

Haemonchus contortus, a parasitic nematode of small ruminants presents significant problems to the international farming industry which are confounded by multi-anthelminthic resistance. Better understanding of this parasite and its developmental requirements are a priority for the development of novel therapeutic agents. Parasite specific microRNAs (small non-coding RNAs which regulate gene expression) are potential future anthelminthic targets. However, little is known about microRNAs regulatory systems in parasitic nematodes. Here, we aimed to examine whether *H. contortus* expressed *lin-41*, a mRNA target for *let-7* microRNA. *H. contortus lin-41* (*Hc-lin-41)* was amplified and sequenced, as was its 3′ untranslated region (3′ UTR). Six potential *let-7* binding sites were identified bioinformatically in the 3′ UTR of *Hc-lin-41*. It can be concluded from this that the regulation of *lin-41* mRNA by *let-7* microRNA is a valid avenue for further research in *H. contortus.*

Project 2: Preliminary results from a web-based questionnaire to explore equine demographics and vaccination in Great Britain

Epidemiological investigations into recent outbreaks of equine infectious disease have been hampered by lack of readily available demographic information about the susceptible population. Endemic diseases such as equine influenza and herpes virus continue to cause economic loss and cases of equine tetanus are reported each year despite availability of vaccinations against these diseases. This is the first study to investigate the association between equine demographic variables and vaccination practices.

Our objectives were to describe British equine demographics and vaccination coverage and to identify factors associated with reduced likelihood of vaccination.
Data were collected using a web-based questionnaire for horse owners. Answers from questionnaire participants between 16th of November 2010 and 10th of March 2011 were described and summarised. Univariable analyses of the association between demographic factors and vaccination practices were performed.

Of 4187, respondents, 4094 listed valid British post-codes and 3277 (80%) answered all of the mandatory questions in the questionnaire. The highest percentage (16.3%) of respondents lived in South West England and the majority (73.5%) kept their horses within 2.5 miles of where they lived. Horses most commonly lived on their owners' own premises (31.6%). Most (70.7%) respondents owned and also rode horses. Hacking (leisure riding) was the most popular activity with 93.5% of respondents reporting participation. Horses were imported by 3.5% of respondents and 3.7% travelled internationally. Equine influenza, tetanus and herpes virus vaccination was reported by 90.3%, 95.1% and 9.2% of respondents respectively. Region, type of premises, type of area, the respondent's type of equestrian involvement, international travel, importation, distances travelled to equine events and the respondent's gender and age group were associated with likelihood of vaccination.

This web-based questionnaire was a useful way to obtain information about the British equine population. The identification of factors associated with reduced likelihood of vaccination can facilitate targeting of vaccination and identification of at risk groups in the case of an outbreak of infectious disease.

Project 3: Characterisation of G proteins in myelin isolated from the *rumpshaker* mouse model of Pelizaeus-Merzbacher disease

The *rumpshaker* mutation of the gene *Plp1* ($Ile^{186}Thr$) that encodes the major myelin protein proteolipid protein (PLP) causes dysmyelination and spastic paraplegia or mild Pelizaeus Merzbacher disease in man. The identical mutation occurs spontaneously in mice. The pathogenic pathway between the *rumpshaker* mutation and dysmyelination has not been elucidated. Phosphotidylinositol 4,5-bisphosphate (PIP2) has recently been shown to influence the stability of the myelin basic protein (MBP) that is critical for competent myelin formation. In a preliminary study, PIP2 levels have been found to be reduced in *rumpshaker* mice. Since $G_{\alpha q}$ is known to initiate phospholipase C mediated hydrolysis of PIP2 this raises the possibility that aberrant G-protein activity may be involved . In the first study to measure G-proteins in *rumpshaker* myelin, we found $G_{\alpha q}$ and $G_{\alpha o}$ levels to be significantly elevated in myelin from C3H *rumpshaker* mice. These findings implicate G proteins as important mediators in a pathway linking mis-folded PLP to MBP dysfunction and may underpin the mechanisms causing dysmyelenation in the *rumpshaker* mouse.

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Acknowledgements

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Project 1

I am immensely grateful to my supervisors Dr Buddhini Samarasinghe, Dr Alan Winter, Dr Collette Britton, and Professor Eileen Devaney for their mentorship.

Project 2

This study used data from a horse owners' questionnaire that was conceived and designed by Dr. Lisa Boden, Dr. Tim Parkin, Professor Rowland Kao, Professor Dominic Mellor and Ms. Julia Yates. The Department for Environment, Food and Rural Affairs funded this work. I would like to thank my supervisors Dr Lisa Boden, Dr. Tim Parkin and Professor Rowland Kao for their mentorship. I also wish to thank the respondents to the questionnaire who made the research possible.

Project 3:

I am very grateful to Dr Mark McLaughlin for his mentorship in this project. I am also grateful to Professor Graeme Milligan for the gift of anti-bodies and to Mrs. Jennifer Ann Barrie for sample preparation. The members of the Applied Neurobiology group Dr Julia Edgar, Mrs Intan Nur Fathia Shaife, Maj-lis McCulloch and Ms Gemma Thompson gave me valuable advice and support in the lab.

Author’s declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.
Signature:

Miriam Casey

List of abbreviations

Project 1

Ce = *Caenorhabditis elegans*

Hc = *Haemonchus contortus*

PCR = polymerase chain reaction

3′ UTR = 3′ untranslated region

Project 2:

AHS = African Horse Sickness

CI = Confidence interval

DEFRA = Department of the Environment, Food and Rural Affairs

EIA = Equine Infectious Anaemia

EIV = Equine Influenza Virus

EHV = Equione Herpes Virus

OR = Odds Ratio

Project 3:

ANOVA = One way analysis of variation

ASPA = Aspartoacylase

DM20 = 26.5kDa protein isoform encoded by *Plp* gene

ECL = enhanced chemiluminescent substrate

G protein = Guanine ucleotide binding protein

HSP 90 = Heat Shock Protein 90

MAG = Myelin Associated Glycoprotein

MBP = Myelin Basic Protein

PMD = Pelizeus Merzbacher Disease

PLP = proteolipid protein (30kDa protein isoform encoded by *Plp* gene).

PLP1 = Proteolipid protein (human gene)

Plp1 = Proteolipid protein (non-human gene)

SDS PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis

3XSDS/DDT 3x concentration sodiumdodecylsulphate/dithiothreitol

General introduction

This thesis describes projects in three different fields; molecular biology, epidemiology and cell biochemistry. Whilst centred around three diverse topics, the projects' common aim is to address an animal or human disease problem through a novel approach. The spectrum of the projects is consistent with the aims of the scientific community to address disease challenges from the level of the molecule to the level of a whole population.

The first project investigates a micro RNA regulatory system in the parasitic nematode of sheep, *Haemonchus contortus*. Multi-drug resistance in parasites is a major problem for the global livestock industry, with *H. contortus*, a parasite of sheep threatening the industry due its resistance to all major anthelminthics. The first project contributes towards using microRNA findings in the model organism *Caenorhabitis elegans* as a possible avenue to control *H contortus*. Little is known about microRNAs in parasitic nematodes and hence our aim was to characterise a microRNA regulatory system, the *let-7* microRNA and *lin-41* mRNA that is well described in C elegans, in its clade V relative, *H contortus*. The hypothesis was that the microRNA target, *lin-41* and potential *let-7* microRNA binding sites on the 3′ untranslated region of this mRNA would be present in *H contortus*.

The second project is a study of British equine demographics and the vaccination practices of horse owners and carers. Recent outbreaks of equine infectious anaemia in Europe and equine influenza in Australia as well as the threat of African Horse Sickness have highlighted the need for more complete demographic information about the equine population to be at hand in order to be prepared for future disease outbreaks. Relative to food animals, equine identification and demographic knowledge in Britain is incomplete. The second project in this thesis is the preliminary analysis of a web-based questionnaire of horse-owners with over 4000 respondents. The questionnaire aimed to collect demographic information about the British equine population relevant to the spread of infectious disease. As well as summarising questionnaire responses, objective of the project was to analyse demographic factors associated with increased or reduced likelihood of vaccination against equine diseases.

The third project is a cell biochemistry project based on measuring G protein levels in the *rumpshaker* mouse model of Pelizeus Merzbacher Disease (PMD), a debilitating congenital

dysmyelinating disorder of the central nervous system. At present, there is no cure for PMD. It is known that mutations in the proteolopid protein gene (*PLP-1*) cause the disease but it is not known how the mutation results in the end phenotype. The *rumpshaker* mouse contains the identical mutation to humans with the mild form of PMD (hereditary spastic paraplegia), Recent studies have linked Phosphatidylinositol 4,5-bisphosphate (PIP2) to myelin basic protein function, which is essential for normal myelination. G proteins are known to effect PIP2 levels. Subsequently, our hypothesis was that G-protein levels would be altered in the myelin of *rumpshaker* mice. This is the first study to investigate G proteins in *rumpshaker* myelin.

Chapter 1: A step towards characterising a microRNA regulatory system in the parasitic nematode *Haemonchus contortus*

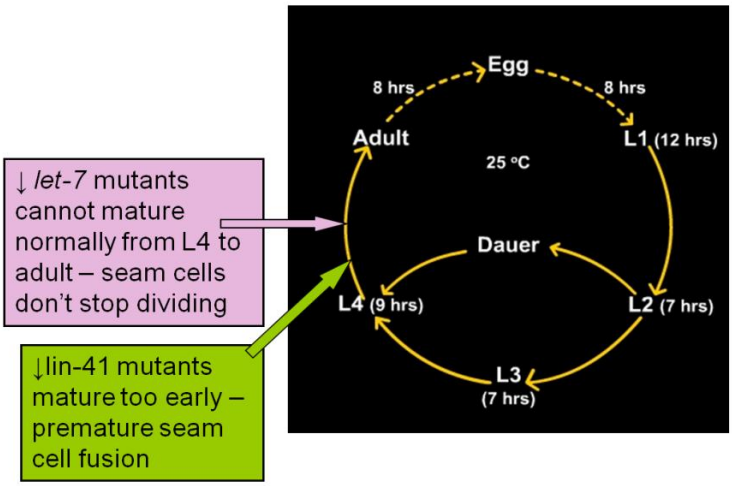
1.1 Introduction:

Haemonchus contortus is a parasitic nematode of sheep and goats of global economic significance. In 2005, intestinal parasitic nematodes of sheep (predominantly *H. contortus* and *Teladorsagia circumcincta*) were estimated to cost the UK economy alone £84M (Nieuwhof and Bishop, 2005). It is an aggressive blood feeder in the abomasum, causing anaemia, diarrhoea and weight-loss in its host. Together with other trichostrongyloid parasites such as *T. circumcincta* and equine cyathastomes, *H. contortus* exhibits a worrying array of resistance to a spectrum of anthelminthics, including benzimadazoles, tetrahydropyramidines/imidithiazoles and macrocytic lactones (Echevarria *et al.,* 1996; Waruiru *et al.,* 1998). Triple-resistant parasites have been reported internationally, including in Scotland. Native breed resistance (Chaudary *et al.,* 2007; Miller *et al.,* 1998) and narrow spectrum novel drugs such as closantel and monepantel offer some hope for the management of haemonchosis. However, economic considerations preclude the use of native low productivity tropical breeds in many countries. Furthermore, the alacrity with which resistance developed to macrocylic lactones which were initially thought to be the "magic bullet" of anthelminthic treatment, mean that development of novel therapies and effective use of existing drugs is a priority.

MicroRNAs are endogenously produced, small, non coding RNAs that regulate the translation of mRNA. They commonly (but not always) bind to the 3′ untranslated region of the mRNA that they are regulating and exert a negative effect on the translation of that mRNA (Vella and Slack, 2005). The first microRNA, *lin-4*, was discovered by Lee *et al.*, (1993) in the free living nematode and model organism *Caenorhabditis elegans*. The discovery of *let-7,* a second microRNA involved in the post-transcriptional regulation of heterochronic genes in *C.elegans* by Reinhart *et al.,* (2000) simulated great interest in micro-RNAs. *let-7* microRNA was found to be highly conserved in many species, including humans

(Pasquinelli *et al.,* 2000), engendering much research into *let-7* and its targets. Whilst the 3′ UTRs of five different heterochronic genes have regions that were complementary to *let-7*, only the negative regulation of *lin-41* by *let-7* during the L3 and later stages appear to impact the normal progression of the life cycle of *C.elegans,* as shown in Figure 1.1 (Reinhart *et al.,* 2000).

Figure 1.1: The role of let-7 microRNA in the control of late temporal transitions in the development of C.elegans. In the fourth larval stage, the seam cells of *let-7* mutant *C.elegans* fail to fuse normally and continue to proliferate, eventually causing vulval bursting. *C. elegans lin-41* mutants have the opposite phenotype, with premature fusion of the seam cells (Reinhart *et al.*, 2000).



Subsequent to the discovery of microRNAs in *C. elegans,* a myriad of microRNAs and microRNA targets have been discovered, with 30% of human genes potentially being regulated by microRNAs (Rajewsky 2006). The role of microRNAs in resistance of neoplasms to chemotherapeutic agents is well documented (reviewed by Ma *et al* .(2010) and Zheng *et al.*,(2010)). Furthermore, recent work on *C.elegans,* indicates that microRNAs may also play a role in drug resistance in nematodes (Simon *et al.*, 2008). Rao *et al.,* (2009) found that a single nucleotide polymorphism in the 3′ UTR of a gene encoding ion channels in *H. contortus* to be associated with ivermectin resistance and postulated that this was at a microRNA binding site. A recent review proposed the investigation of microRNA regulation of both drug targets and drug evasion mechanisms in *H. contortus* as a logical but novel approach to the problem of anthelminthic resistance in parasitic nematodes (Devaney *et al.,* 2010).

Like *C. elegans*, *H. contortus* is a Clade V nematode and mechanisms regulating development, such as microRNAs and their targets, may be conserved. In order to pursue microRNAs or their binding sites as potential drug targets in *H. contortus*, their presence and action in the worm must be validated. Due to much cross-species research, *let-7* is the best understood microRNA. The regulation of the hetero-chronic gene *lin-41* by *let-7* has been extensively studied in *C. elegans*. Consequently, this is the ideal microRNA system with which to begin investigations into microRNA activity in *H. contortus*. This study aims to contribute to the understanding of microRNAs in *H. contortus* by yielding information about the *let-7* target *lin-41 mRNA.* Specifically, the objectives of the study are to confirm the presence of *Hc-lin-41*, to determine its nucleotide sequence and to determine the nucleotide sequence of 3′ UTR of *Hc-lin-41* and seek *let-7* complementary sites on this 3′ UTR.

1.2 Materials and methods:

1.2.1 cDNA production from an adult population of H. contortus

Total RNA was extracted from a mixed sex population of adult *H. contortus* harvested from a single infected sheep after 28 days of infection (kindly provided by the Moredun research institute and Dr Buddhini Samarasinghe) and cDNA was produced from this using a commercial protocol (Affinity Script multiple temperature cDNA synthesis kit, Stratagene).

1.2.3 Bioinformatics

The publically available nucleotide and translated sequences for *Ce* were obtained from Wormbase" (www.wormbase.org). Available known *Hc* supercontigs were obtained from the Sanger institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/h_contortus). For nucleotide and translated sequence comparison, alignment and primer design, the publically available Basic Local Alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the bioinformatics programme Vector NTI (Invitrogen) were used. For seeking out potential *let-7* microRNA complementary sites, RNA Hybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html) was used. Where nucleotide sequence was translated to amino acid sequence, the Prosite proteomics programme (http://us.expasy.org/tools/dna.html) was used. Peptide motifs also were identified in translated sequences and diagrammatically represented using the Prosite programme (http://expasy.org/prosite/).

1.2.3 PCR to amplify the 5' end of Hc-lin-41, full-length Hc-lin-41and Hc-lin-41 3' UTR

Oligonucleotide primers were synthesized commercially by Eurofins MWG Operon. Templates, primers and cycle times used for each PCR are described in Table 1.1. Amplification of *Hc-sod-1* (Liddell and Knox 1995) *and Hc-actin-1* (primer sequences from Dr. Buddhini Samarasinghe) was used as PCR controls. Products were visualised on 1% agarose gels containing 0.003% ethidium bromide (10mg/ml).

To amplify the full length *Hc-lin-41,* a product containing a high fidelity polymerase (PFU turbo, Stratagene) was used. The high-fidelity polymerase was added to reduce the chance of error in the amplification of a long DNA fragment.

After the 3′ end of *Hc-lin-41* was identified, the 3′ RACE technique was used to amplify the 3′ UTR of this gene. Two gene specific forward nested primers from the 3′ end of *Hc-lin-41*

and the Abridged Universal Amplification primer (AUAP) were used in the Invitrogen protocol for 3′ RACE (Table 1.1).

Table 1.1: PCR conditions for the amplification of the 5′ end of *Hc*-lin-41, full-length and *Hc*-lin-41 3′ untranslated region.

cDNA amplified	Template	Primers	Pol	Cycle stage	Temp	Time	Cycles
5 ′ portion of *Hc-lin-41*	*Hc*-cDNA	SL-1, *Hc-lin-41* -R1	Taq	Denaturation	94°C	3 m	1
				Denaturation	94°C	30s	
				Annealing	55°C	30s	30
				Extension	72°C	3m	
				Extra extension	72°C	5m	1
Full length *Hc-lin-41*	*Hc*-cDNA	*Hc-lin-41* Forw, Rev	PFU turbo	Denaturation	94°C	3 m	1
				Denaturation	94°C	1m	
				Annealing	55°C	1m30s	30
				Extension	72°C	8m	
				Extra extension	72°C	5m	1
3′ RACE primary PCR *Hc-lin-41* 3′ UTR	*Hc*-cDNA	*Hc-lin-41*-F3, AUAP	Taq	Denaturation	94°C	3 m	1
3′ RACE, nested PCR for *Hc-lin-41* 3′ UTR	PCR product of *Hc-lin-41*-F3 and AUAP	*Hc-lin-41*-F4, AUAP	Taq	Denaturation	94°C	30s	
				Annealing	55°C	30s	30
				Extension	72°C	2m	
				Extra extension	72°C	5m	1

1.2.4 Cloning of the 5 ' portion of Hc-lin-41, full length Hc-lin-41 and Hc-lin-41 3' UTR

PCR products for cloning and sequencing were extracted from the agarose gel and purified using QIA quick gel extraction kit (http://www.qiagen.com/products/dnacleanup/gelpcrsicleanupsystems/qiaquickgelextractionkit.aspx). Where products had been amplified with Taq polymerase, the purified DNA was ligated to pSC-A-amp/kan cloning vectors containing the Lac Z (beta-galactoside) gene (Strataclone PCR cloning kit (http://www.genomics.agilent.com/files/Manual/240205.pdf). A competent cell line (from Strataclone PCR cloning kit) was transformed with these vectors and was cultured on Luria-Bertani Broth (LB) agar containing ampicillin and a marker of beta-galactoside activity, X-gal. After overnight incubation at 37°C, colonies that had been transformed by successfully ligated vectors (as evidenced by lack of beta-galactoside activity shown by blue-white selection) were sampled. DNA was extracted from the samples using the QIAprep Spin Miniprep kit (http://www.qiagen.com/products/plasmid/qiaprepminiprepsystem/qiaprepspinminiprepkit.aspx). Colony PCR was performed with the primers used in the original PCR. Colonies corresponding with amplification of DNA fragments of the same size as the original PCR products were inoculated overnight in large volume tubes containing LB. Plasmid DNA from these colonies was then purified using the QIAprep Spin Miniprep kit. The DNA yielded from plasmid purification was subjected to spectrophotometry at a wavelength of 260nm to determine its concentration. The concentration of samples selected for commercial sequencing was adjusted to 100ng/microlitre.

Where *Hc-lin-41* was amplified with PFU Turbo polymerase, which does not add poly-A tails to PCR products, gel extracted DNA was adenylated following the protocol from Strataclone PCR cloning kit before being ligated to pSCA-A vectors and subjected to the cloning protocol described above. Due to the extended PCR that was required for amplification of the potential *Hc-lin-41* fragment (Table 1.1), the presence of *Hc-lin-41* cDNA in the plasmid DNA was tested with a PCR using primers (*Hc-lin-41*-F2, R1, table 1.2) for a 300bp length from a previously sequenced portion of the potential *Hc-lin-14* rather than primers for the full length of the gene.

Prior to commercial sequencing, in order to confirm the presence of the *Hc-lin-41*insert, plasmid DNA was subjected to a restriction digest using EcoRI which is a restriction site on

both TOPO and psc-A vectors. The products of the restriction digest were visualised on 1% agarose gels containing 0.003% ethidium bromide.

Dideoxynucleotide sequencing was performed commercially by Eurofins MWG Operon.

1.3 Results:

1.3.1 Alignment of C. elegans LIN-41 with available translated H. contortus supercontigs

The translated *Hc* supercontig 0048734 contained three areas which had a high degree of sequence identity to amino acid sequences near the C terminal of *C. elegans* LIN-41. These three high scoring pair (HSP) areas were 122, 174 and 1861 nucleotides in length with 48%, 73% and 85% identical amino acid matching (respectively) and 67%, 90% and 91% 91% similar amino acid matching. Primers were designed from these areas (Figures 1.2, 1.3 and Table 1.2). As the chosen HSP areas aligned near the C terminal of *Ce* LIN-41, the primers designed from them were deemed likely to be complementary to the 3′ end of the hypothetical *Hc-lin-41* cDNA. No alignment between translated *Hc* supercontigs and the N terminal of *Ce* LIN-41 was achieved (Figure 1.2).

Figure 1.2: Alignment of Caenorhabditis elegans LIN-41(Ce LIN 41) and potential translated Haemonchus contortus lin-41 mRNA. The high scoring pair areas of alignment between *Haemonchus contortus* supercontig 004874 and *Ce* LIN-41 were located near the C terminal of *Ce* LIN-41.

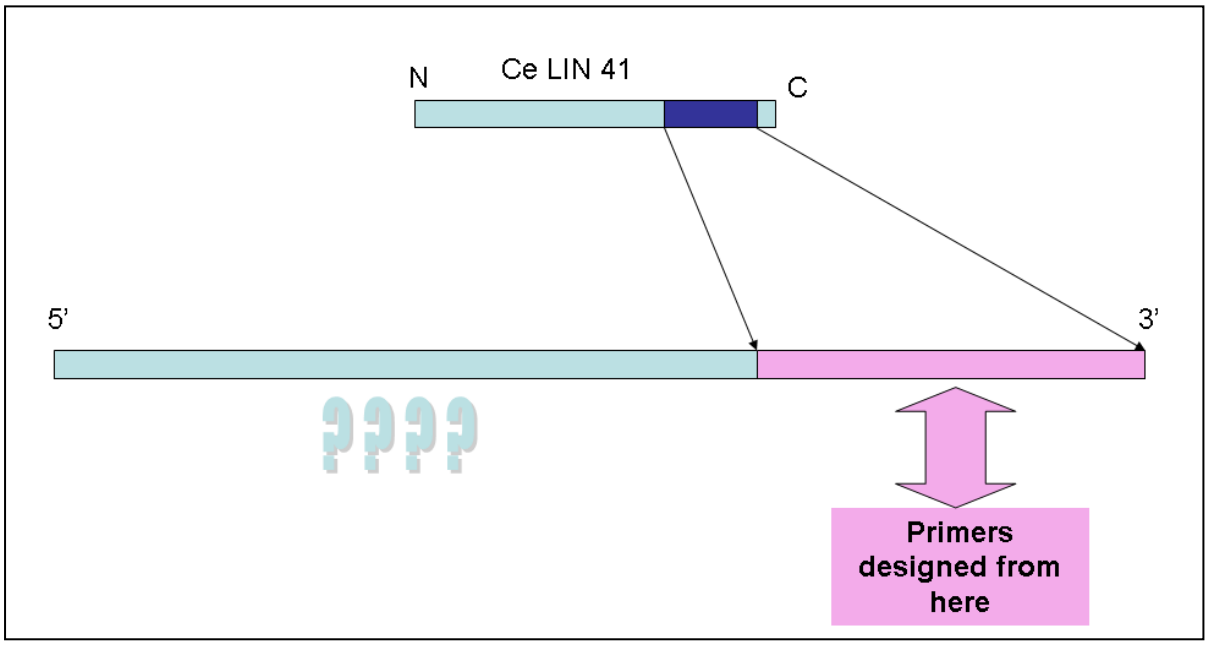


Figure 1.3: Primer locations on Haemonchus contortus supercontig 0048734. Primers were designed from the high scoring pair areas that aligned near the C terminal of *Ce* LIN 41. The location of the stop codon on the supercontig is also highlighted. This is drawn to approximate scale using the "Vector NTI" alignment programme.

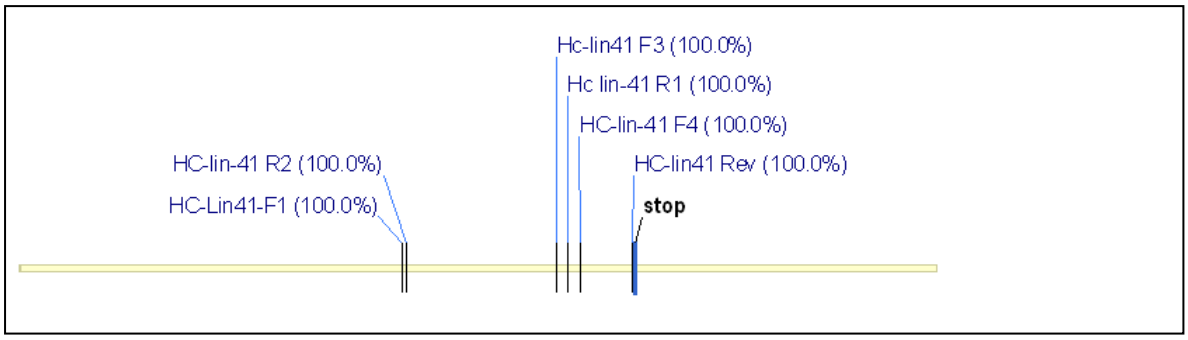


Table 1. 2: Nucleotide sequences of primers designed from Haemonchus contortus supercontig 0048734. Except for *Hc-lin-41* –Rev all primers shown in the table were designed from the high scoring pair (HSP) areas on the super-contig that aligned with *Caenorhabditis elegans* LIN-41. *Hc*-lin-41-Rev was designed from an exon in *Haemonchus contortus* supercontig 0048734 beside the stop codon nearest the HSP areas that aligned with *Caenorhabditis elegans* LIN-41).

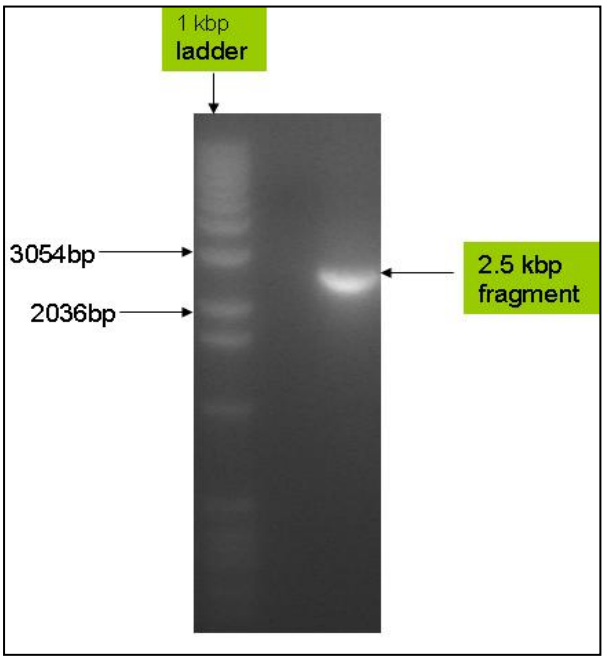
Primer Name	Primer Sequence (5′ →3′)	Corresponding nucleotides on *Ce* lin 41 (total length 3444bp)
Hc-lin-41 F2	CAATATTGTGGTAGCCGATAAGG	2703-2726
Hc-lin-41 F1	GTGGTAGCCGATAAGGACAACC	2713-2736
Hc-lin-41 R2	CCGTTCTCATCGAACACTTGG	2771-2750
Hc-lin-41 F3	CAGGTATTCAATGAAAATGGCC	2892-2914
Hc-lin-41 R1	GGAGTTGACCATCAGATAGG	3007-2987
Hc-lin-41 F4	CGTTTGGCCGTGCTGTCCTCTCG	3030-3053
Hc-lin-41 Rev	TTAGAAAACGCGGATGCAATTGTTGCCG	Exon near stop codon nearest to HSP areas

1.3.2 Amplification of the 5' end of Hc-lin-41

A primer was designed based on the trans-spliced sequence "Spliced leader 1" (SL-1, GGTTTAATTACCCAAGTTTGAG) that is known to be present on 70% of the 5'ends of all *C. elegans* mRNA (Blumenthal and Steward, 1997). A DNA fragment of 2.5kbp in size was amplified in PCR using adult *Hc* cDNA and the primers SL-1 and *Hc*-lin41-R1 (Figure 1.4). As a control, PCRs were performed under the same conditions using SL-1 alone and R1 alone. These control PCRs did not amplify cDNA fragments. This 2.5kb fragment was reamplified, purified, cloned, sequenced and analysed bioinformatically. Sequences of 630bp in length were identified for DNA adjacent to the SL-1 and *Hc*-lin41-R1 sequences. Upon BLAST with known *Hc* supercontigs, the identified sequences aligned perfectly with portions of *Hc* supercontig 0048110.

As the SL-1 primer was a non specific primer, a gene specific forward primer was needed from the sequenced *Hc-lin-41*. Amplification of 5' end of *Hc-lin-41* with this new primer replacing the SL-1 primer increased confidence that the amplified fragments were the 5' end of *Hc-lin-41* rather than erroneously amplified fragments. As the sequenced area adjacent to the SL-1 sequence was potentially the 5' end of *Hc*-lin-41, the new gene specific primer, *Hc*-lin-41-Forw (ATGCAGCGAGTGGATTCCCGAAGCG), was designed from here.

Figure 1.4: Amplification of the 5′ PCR products. A PCR using adult Haemonchus contortus cDNA. The primers SL-1 and *Hc*-lin41-R1 and Taq polymerase amplified DNA fragments of 2.5kbp in size (shown here on an ethidium-agarose gel).



1.3.3 Amplification of the full length of Hc-lin-41

A PCR using adult *Hc* cDNA, *Hc-lin-41*-Forw and *Hc-lin-41* Rev primers and "PFU turbo" high fidelity polymerase amplified DNA fragments of just under 3kbp in size (Figure 1.5). This was reamplified, purified, cloned, sequenced and analysed bioinformatically. This yielded nucleotide sequences of 629 and 700 bp in size from the 5' and 3′ ends of the potential *Hc-lin-41* respectively. These sequences were aligned with the sequences from the SL-1 and R-1 product and showed perfect homology.

Primers, *Hc-lin-41*-R3 and *Hc-lin-41*-F5, for internal sequencing of the full length *Hc*-lin-41 cDNA fragment were designed based on the end of the sequenced portions of the potential *Hc-lin-41*. These were used with the purified *Hc-lin-41* plasmid DNA to sequence the remainder of *Hc*-lin-41 (Table 1.3).

Figure 1.5: Amplification of full length Hc-lin-41. A PCR using Haemonchus contortus adult cDNA. the primers *Hc*-lin-41-Forw and Rev and PFU turbo polymerase amplified DNA fragments of just under 3kbp in size (shown here on an ethidium-agarose gel).

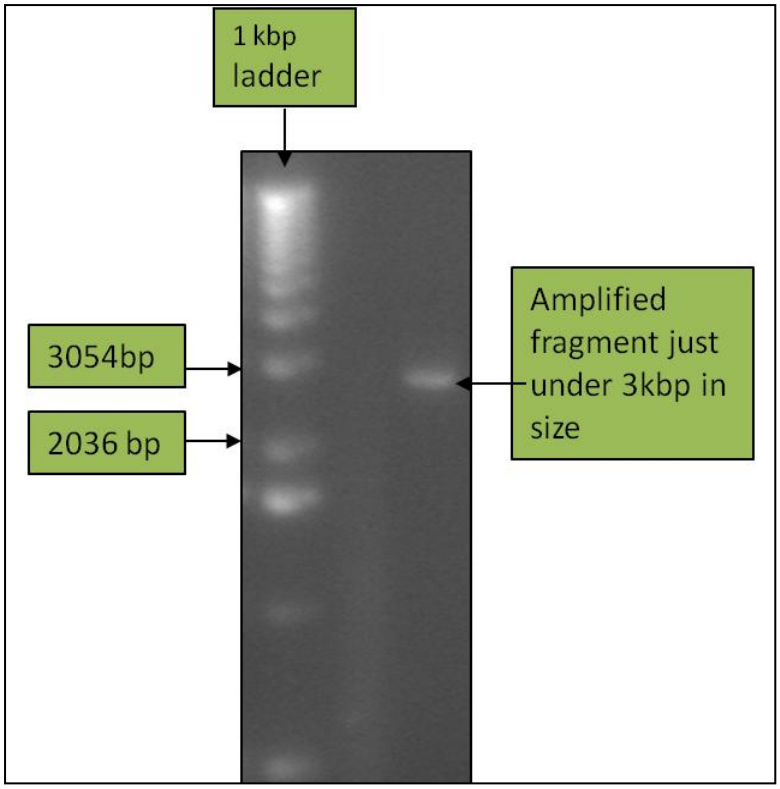


Table 1.3 : Hc-lin-41-R3 and Hc-lin-41-F5 primers. These were designed based on the ends of the sequenced portions of the potential *Hc-lin-41* for internal sequencing of this DNA fragment.

Primer name	Primer sequence	Origin
HC lin-41-R3	CAC GCC TGG GTC GCT TTC GCT TCG TC	From sequenced *Hc*-lin-41-R1 portion
HC lin-41-F5	GTCTGAGCGATCTGTCCGGTCATTTG	From sequenced SL-1 portion

1.3.4 Bioinformatic analysis of full length Hc-lin-41

All sequenced portions of *Hc*-lin-41 were then aligned with to identify a full length gene of 2871 bp in size. This was translated to *Hc* LIN-41 amino acid sequence (Figure 1.6) and compared to *Ce* LIN-41. BLAST indicated 41% identical matches and 55% similar matches. There was a far greater density of high scoring pairs near the C terminals of *Hc* LIN-41 and *Ce* LIN 41. i.e. when the half of *Hc* LIN-41 adjacent to its C terminal was aligned with *Ce* LIN 41, there were 61% and 75% identical and similar matches respectively. Protein motifs were identified in *Hc*-LIN-41 sequence and compared to human and *Ce*-LIN-41. These three species had a Zinc-finger B-box motif near the centre of their open reading frames. They also expressed filamin repeats and 6 repeat NHL motifs near their C termini, whilst a zinc finger ring motif was expressed uniquely at the N terminus of human LIN-41 (Figure 1.7).

Figure 1.6: Alignment of *H. contortus* LIN-41 and *C. elegans* LIN-41. High scoring pair areas are concentrated towards the C terminals of both translated sequences. Overall identical amino acid matches were 41% and similar matches were 55%. However, when the half of Hc LIN-41 adjacent to its C terminal was aligned with Ce LIN 41, there were 61% and 75% identical and similar matches respectively.

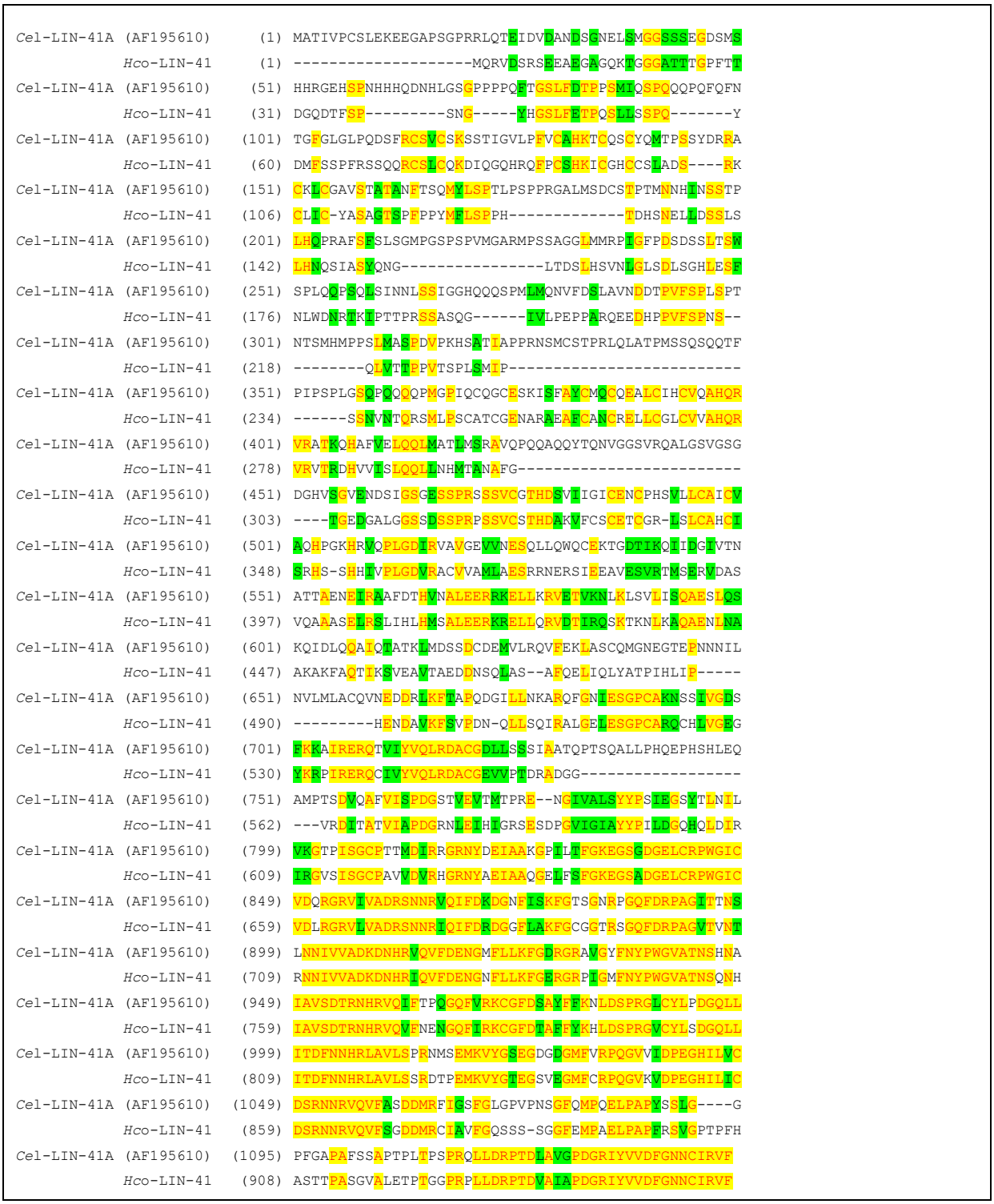
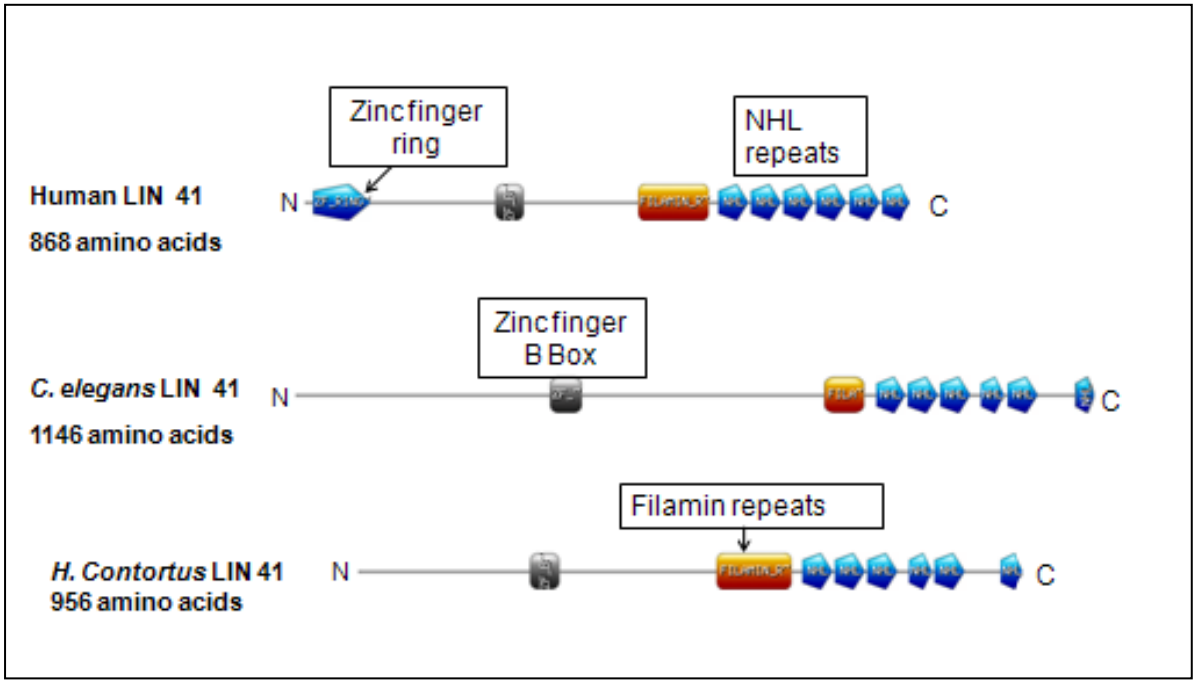


Figure 1.7: A comparison of the peptide motifs of human, C. elegans and H.contortus LIN-41. These motifs were searched for and graphically represented in the Prosite programme. Human and C. elegans LIN-41 sequences were obtained from the NCBI website. All three species share filamin and six NHL repeats near their C terminals as well as a Zinc finger B-Box motif in the centre of their LIN-41 sequences. A zinc finger ring motif was recognised at the N terminal of human LIN-41 alone.



1.3.5 Identification of the 3′ UTR of Hc-lin-41 using 3' RACE and identification of potential let-7 binding sites

Using AUAP and gene specific primers*Hc*-lin-41 F3, 3′ RACE amplified a fragment of 1.6kbp size (Figure 1.8). This was cloned, sequenced and analysed bioinformatically to identify nucleotide sequences of 645 and 627 bp in length adjacent to the F4 and AUAP primer sequences respectively. These sequences were aligned with *Hc* supercontig 0048734 and found to have excellent sequence identity with either end of a 1659bp portion of the supercontig (Figure 1.9 and appendix). Given that the amplified fragment was 1.6kbp in size, and its high degree of sequence identity with the sequenced portions of the *Hc*-lin-41 3′ UTR, this portion of the supercontig was accepted to be the 3′ UTR of *Hc*-lin41. *Hc-let-7* microRNA binding sites were sought this portion of the supercontig using the "RNA Hybrid" programme. Seven possible binding sites for *let-7* were found. Table 1.4 lists these sites and shows the imperfect binding which is a feature of microRNA target binding. The conformation imposed by this imperfect base pairing between the microRNA and its target is represented in Figure 1.10. One of the binding sites at 303 on the supercontig portion was before the stop-codon, meaning it was on the *Hc-lin-41gene* rather than the 3′ UTR.

Figure 1. 8: Amplification of the 3′ UTR of Hc-lin-41. Using the nested primers *Hc-lin-41*-F3, -F4 and the abridged universal amplification primer (AUAP), a 3′ RACE amplified fragments of 1.6kbp size.

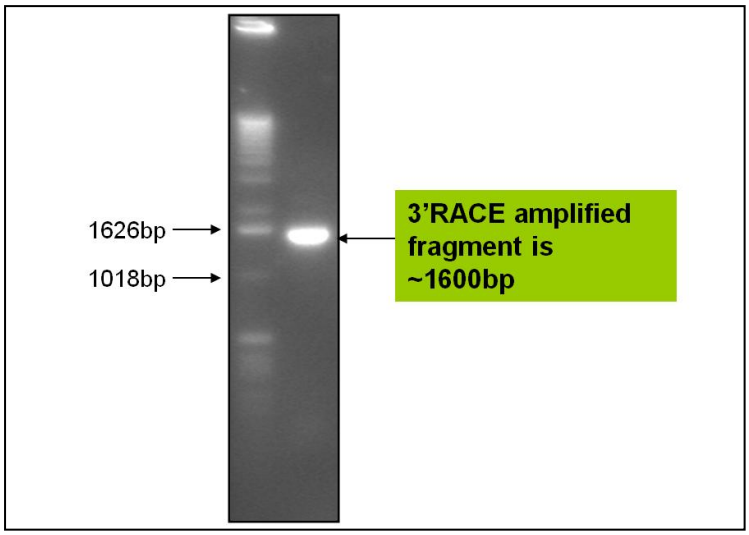


Figure 1.9: Alignment of the nucleotide sequences from DNA fragments amplified with 3′ RACE with Haemonchus contortus supercontig 0048734. This diagram was created using the "Vector NTI" alignment programme and is approximately to scale. The circles represent potential *let-7* microRNA binding sites identified on the portion of *Hc* supercontig 0048734 which aligned with the sequences. The triangle represents the stop codon and the beginning of the 3These were drawn onto the diagram using a graphic programme (Gimp). The red dot represents the stop codon for the *Hc-lin-41* gene.

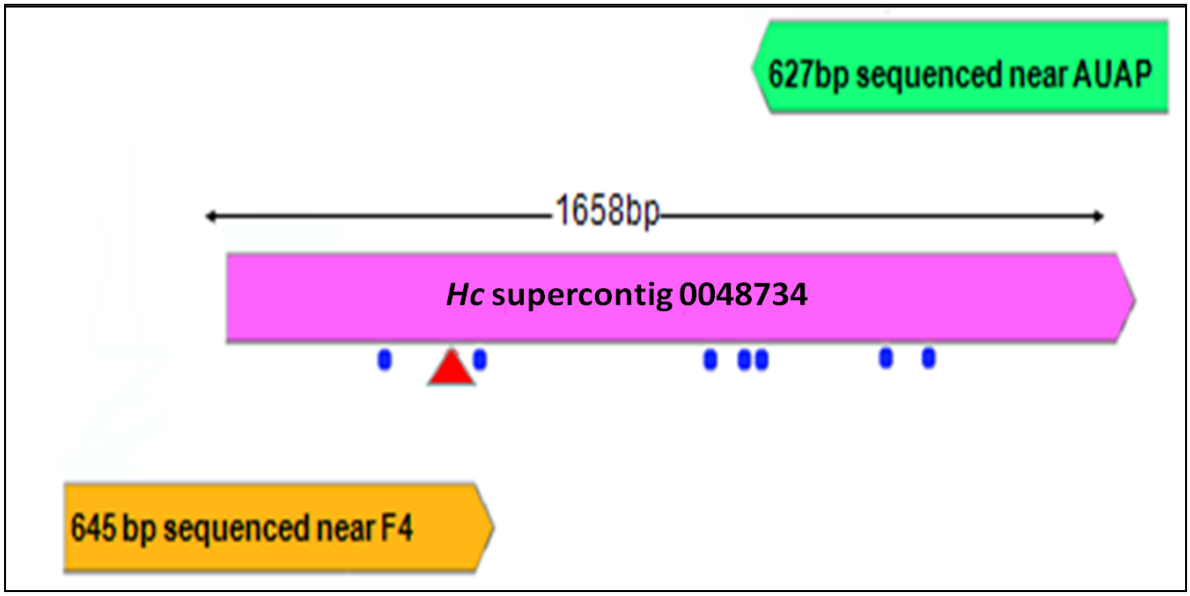


Table 1.4: Locations of *let-7* complementary sites on the portion of Hc supercontig 0048734 which aligned with the sequenced portions of the 3′ UTR of Hc-lin-41. (*Hc* = Haemonchus contortus, mfe= minimum folding energy). The "seed" refers to the portion near the 5′ end of the microRNA that binds to the 3′ end of the target sequence with perfect complementarity. Imperfect base pair matching is a feature of other portions of the microRNA target interaction. The likelihood of a true interaction is based on the mfe and the length of the area perfect complementarity.

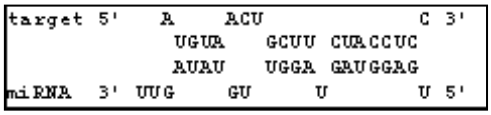
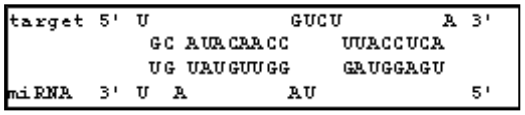
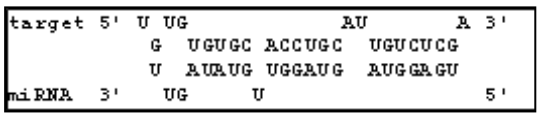
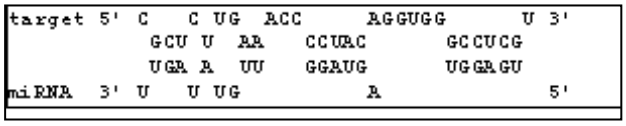
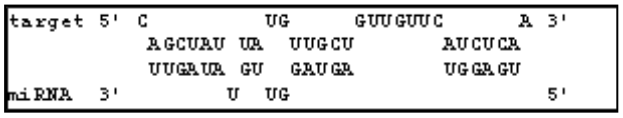
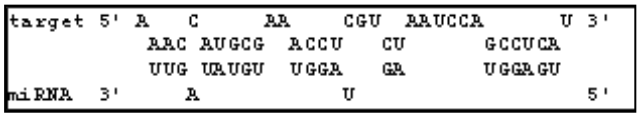
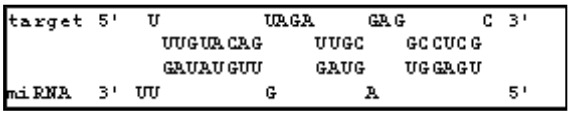
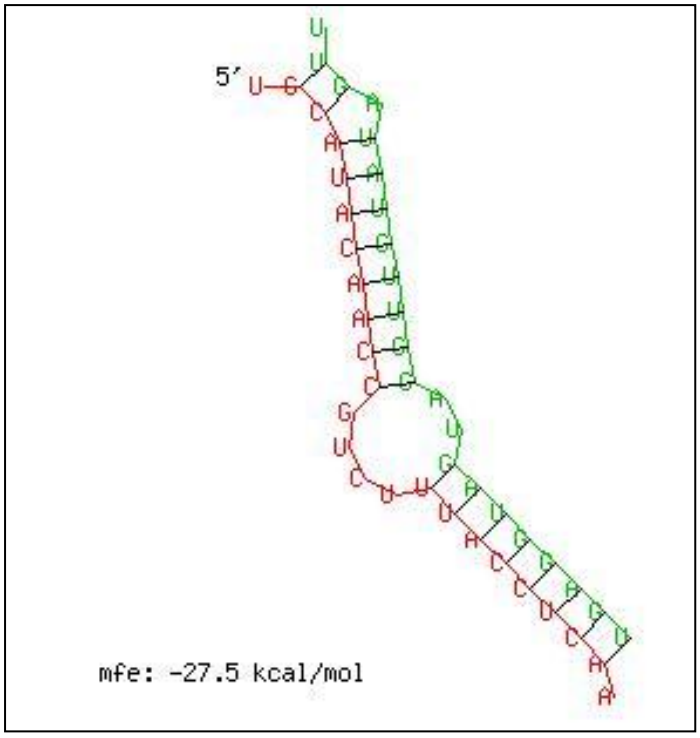
Position on 1658bp *Hc* supercontig portion	*let-7* - *Hc-lin-41* 3′ UTR interaction	Mfe (kcal/mol)	Seed length (bp)	Likelihood of true interaction
881		-21.1	7	Strong
952		-27.5	8	Strong
1291		-23.1	7	Strong
303		-21.4	6	Not on 3′ UTR
464		-20.8	6	Medium
974		-21.5	6	Medium
1211		-22.5	6	Medium

Figure 1.10: A representation of *let-7* (microRNA) – *Hc-lin*-41 (target) interaction at site 952 on the 3′ UTR of Hc-lin-4. This was created on the RNA Hybrid programme.



1.4 Discussion

We can conclude from our findings that *Hc* expresses the gene *lin-41* and that the 3′ UTR of this gene contains potential *let-7* microRNA binding sites.

1.4.1 Use of the SL-1 primer

The use of the trans-spliced SL-1 sequence to amplify the 5' end of *Hc-lin-41* was a useful technique and saved reverting to 5' RACE or other methods to amplify the unknown sequence. The SL-1 sequence was initially discovered by Murphy *et al.,* (1986) in the mRNA of *Trypansoma brucei* and shortly afterwards by Krause and Hirsh (1987) in *C.elegans*. This sequence is now recognised to reside at the 5' end of 70% of all *C. elegans* mRNA (Blumenthal and Steward 1997). SL-1 is also reported to be trans-spliced to the 5' end of *H. contortus* mRNA as well as other parasitic nematodes (Bektesh *et al.,* 1988) and has been used to great advantage in studies where sequencing of *Hc* mRNA or cDNA was required (Rowe *et al.,* 2008). However, SL-1 is not a gene specific primer. For this reason, once the SL-1 technique yielded a potential sequence for the 5' end of *Hc*-lin-41, it was necessary to repeat the sequencing using a primer designed from the newly sequenced 5' end. The amplification of t full-length *Hc-lin-41* which, upon translation, had good sequence identity with *Ce* LIN-41 illustrates that this methodology utilising the SL-1 primer was effective in amplifying the gene.

1.4.2 H. contortus LIN-41 sequence identity with other species

The high degree of sequence identity between the C terminal ends compared with the N terminal ends of *Hc*-LIN-41 and *Ce*-LIN-41 is similar to the case in vertebrates (Schulman *et al.,* 2005) *and Brugia malayi* (Ghedin *et al*., 2007, Dr Alan Winter, unpublished data). The excellent conservation of peptide motifs in *H. contortus, C. elegans* and human LIN-41 (Figure1.7) at the C terminal end with an absence of shared motifs at the N terminal end is consistent with the divergence of amino-acid sequences at N termini. It explains why initial alignment comparing *Ce* LIN-41 and known translated *H. contortus* supercontigs did not identify any areas with high identity near the N terminus. This absence of conserved motifs near the N terminus of LIN-41 across species suggests reduced evolutionary pressure for conservation at this site.

1.4.4 Let-7 complementary sites in the 3′ UTR of Hc- lin-41

The presence of six *let-7* complementary sites on the 3′ UTR of *Hc-lin41* is a positive start for investigation into *let-7* target interaction in *Hc*. However, it should be borne in mind when considering this result that potential *let-7* binding sites identified bioinformatically are not evidence of functional *let-7* – target interaction. For example, whilst the 3′ UTRs of five different heterochronic genes have regions that were complementary to *let-7*, only the negative regulation of *lin-41* by *let-7* during the L3 and later stages appear to impact the normal progression of the life cycle of *C.elegans* (Reinhart *et al.,* 2000). There have been similar findings with other microRNAs that bind specific targets with great effect but to a broad range of further targets with little biological effect. It is believed that these targets are acting as microRNA "sponges," lessening the effect of the microRNAs on their "action" targets (Kaufman and Miska, 2010).

Bioinformatic techniques have been shown to identify thousands of complementary sites for some microRNAs (Miranda *et al.,* 2006). Furthermore, in *C. elegans* alone there are 175 recognised microRNAs (www.mirbase.org). Of these microRNAs, only eight families have validated target interactions (Kaufman and Miska 2010). A recent review highlighted the necessity of *in-vivo* verification of microRNA functions and pathways through genetic knockout and study of resultant phenotypes in addition to bioinformatic techniques (Resnick *et al.,* 2010).

1.4.4 Future investigation of let-7 – lin-41 interaction in H. contortus and use of C. elegans as a transgenic model system

The above observations from literature regarding the plethora of remaining elucidations to be made about microRNA target interactions highlight the rationale of basing initial studies in *H contortus* on the best understood microRNA regulatory system, *let-7* and l*in 41*, in *C. elegans*. This maximises the knowledge base from which to initiate investigations into a new species.

Due to the necessity of a host to complete its life-cycle, sexual reproduction and limited response to RNA interference, there are no reliable methods to knock down or over express genes directly in *H. contortus* (Knox *et al.,* 2007; Samarasinghe *et al.,* 2011). However, *C. elegans* has been successfully transformed to express genes from *H. contortus* (Britton *et al.,*

1999). Furthermore, *Ce- let-7* and *Hc-let-7* are identical (Pasquinelli *et al.*, 2000). It may be possible therefore, to perform a transformation of *C. elegans* with *Hc-lin-41-3′ UTR* and a green fluorescent protein gene. Changes in green fluorescent protein could highlight the interaction between *let-7* and *Hc-lin-41-3′ UTR* during the different life stages and verify if *Hc-lin-41-3′ UTR* functions in a similar manner to *Ce-lin-41-3′ UTR*. If the interaction between *let-7* and *lin-41* in *H. contortus* was defined by green floursecent protein expression in transgenic *C. elegans*, it could then be used as a model with which to test methods for parasitic microRNA disruption (e.g. using oligonucleotides to bind to the microRNAs and inhibit their function as described by Kundu and Slack (2010)). Validating such approaches in a trans-genic model would greatly facilitate and expedite selection of the most viable microRNA disruption approach for testing in *H. contortus* itself, making most efficient use of resources and minimising the use of experimental sheep.

Quantitation of *lin41, let-7* and LIN-41 at the different stages of the life cycle of *H. contortus* would also provide valuable information about the relationship between this microRNA and its target in *H. contortus* and the impact on translation of the target mRNA.

In conclusion, our findings confirm that *lin-41* is present in *H. contortus* and that the 3′ UTR of this gene has potential *let-7* binding sites. Subsequently, investigation into the interaction between *let-7* microRNA and *lin-41* mRNA in *H.contortus* is a valid avenue for further research.

Chapter 2: Preliminary results from a web-based questionnaire to explore equine demographics and vaccination in Great Britain

2.1 Introduction

Demographic knowledge of the population at risk greatly facilitates outbreak investigation and epidemiological investigation of any kind. Parameters such as disease prevalence, incidence, measure of the transmission of an infectious agent, by definition, incorporate the population at risk (Trusfield, 1997). Whilst dealing with farm animal disease outbreaks of foot and mouth disease (FMD) and bovine spongiform encephalopathy in Britain, the industry and authorities had the great advantage of detailed demographic and movement information about the populations of food animals at risk. This facilitated rapid investigations into the FMD epidemic (Ferguson *et al*., 2001) and Bluetongue emergence in northern Europe in 2006 and in England in 2007 (Anon, 2007b).

In the case of equine disease, such investigations would be challenging as our knowledge of the equine population, its whereabouts and movements is incomplete (Robin *et al*., 2011). This was highlighted in a commentary on the Equine Infectious Anaemia (EIA) outbreak in Ireland, where the introduction of a standardised approach to horse identification and mandatory registration of all equine premises was called for (Branagan *et al*., 2008). Similar advice was issued in a review of the EIA outbreaks in Britain in 2010 (Anon, 2011). A paucity of knowledge of the equine populations at risk was cited as a factor which increased the difficulty associated with outbreak investigation of equine influenza (EIV) in Australia (Cowled *et al*., 2009). Similarly, if a large scale outbreak of equine influenza or herpes virus was to occur in Britain, it would be very difficult estimate the location and vaccination coverage of the non-thoroughbred population at risk.

Efforts have been made to address the issue of equine identification and movement tracing by making it a legal requirement for every horse, except a few covered by derogations, to have a traceable passport (Anon 2008). However, it is suggested that 25% of horses in the UK do not have a passport (Alison *et al.* 2009) and that some passports may be duplicated or obsolete (Robin *et al*., 2011).

Increased movements of humans, horses and equine products are believed to be the single most important factor in the emergence of trans-boundary equine diseases (Herholz *et al*., 2008; Leadon and Herholz, 2009; MacLachlan and Guthrie, 2010). This is a particularly relevant observation with the planned arrival of elite athletic horses from multiple international locations for the 2012 Olympic Games in London. Furthermore, assessment records of the European Union's Trade Control and Expert System (TRACES) indicate that there has been an increase, especially since 2005, in the extent of global horse movements (Herholz *et al*., 2008).

Despite the availability of vaccines, outbreaks of equine influenza continue to cause economic loss in endemic countries (Elton and Bryant, 2011; Gildea *et al*., 2011) as well as devastating outbreaks naïve populations such as that in Australia in 2007 (Callinan, 2008). Influenza vaccination has been mandatory in competitions affiliated to the Federation Equestre Internationale and British Horse-racing since 1981. However, concerns were cited in a recent review that the majority of non-competition horses in the UK were not vaccinated against influenza (Elton and Bryant, 2011). The results of two recent postal questionnaires reported influenza vaccination by 59% of 1431 horse owners (Hotchkiss *et al*., 2007) and unspecified vaccination by 84% of 1111 of fostered donkey owners (Cox *et al*., 2010). A recent survey of indicated that 66.3% of 918 geriatric horses were vaccinated against influenza. Inadequate vaccination was cited as an important potential risk factor in an epidemiological investigation of 28 influenza outbreaks in Ireland (Gildea *et al*., 2011). With the current situation of epidemics of genetically divergent equine influenza and the ability of influenza viruses to cross species, there is great interest in the susceptibility of the British equine population to infection (Elton and Bryant, 2011).

Equine herpes viruses (EHV) 1 and 4 can cause respiratory disease and EHV-1 is also associated with abortion and neurological disease. There has been an increase in incidence of EHV-1 myeloencephalopathy in the past decade with devastating outbreaks at race tracks, horse shows and veterinary hospitals internationally (Perkins *et al*., 2009). It is considered a potentially emerging infectious disease in the United States (Anon, 2007a) and there are calls to increase population resistance to herpes virus through widespread vaccination.

In the surveys of Hotchkiss *et al*. (2007) and Ireland *et al*. (2011), more horses were vaccinated against tetanus than for flu (82.8% and 67% respectively). However, despite the

strong protective effect of vaccination against tetanus (Holmes *et al*., 2006), cases are still being reported in equine hospitals in countries such as the USA where vaccination is widely available (Green *et al*., 1994). For this reason, a representation of the protection of the British equine population of this fatal but easily preventable disease is necessary.

The unvaccinated subset of the equine population is potentially important in the propagation of endemic infectious diseases such as influenza and herpes virus. To the author's knowledge, there have been no previous investigations into the association between demographic factors and vaccination practices in the general equine population. This information, as well as a general preliminary description of British equine demography could be a valuable addition to the investigative artillery of an epidemiological team in the case of an outbreak of equine infectious disease.

The aims of this present study are to give a preliminary overview of British equine demography and vaccination coverage from the results of an ongoing web-based questionnaire. Specifically, we wish to describe the association between equine influenza, tetanus and herpes vaccination practices amongst horse owners and carers and other demographic variables that we investigated in the questionnaire.

2.2 Materials and methods

2.2.1 Data collection

A cross-sectional study of British horse owners was initiated on the 16th of November 2010 and is currently ongoing. A link to an online 'Horse Owners Questionnaire' was publicised through the equestrian media, relevant equine websites and in e-mail lists from equine organisations. These included the National Equine Database (8,500 members with valid email addresses**)**, World Horse Welfare (25,000 active emails), British Eventing (8,000 members), British Dressage (10,000 members), Endurance GB (1,800 members), BHS (40,000 email contacts, 60,000 website visitors), Horse and Hound (weekly readership 250,000). Questionnaires in English and Welsh were completed using an online survey tool ("survey monkey").

The participating websites and media invited readers to answer the questionnaire relating to horses under their care or management and stipulated that only one person per family answer the questionnaire. The questionnaire contained 30 questions (23 of which were mandatory) relating to use of horses, location, travel, importation, vaccination horse owner age and sex and registration with the National Equine Database. The questions had multiple choice answers, with the opportunity, where applicable, for the respondent to answer "other" and make a qualitative comment relating to this. For some multiple choice questions, respondents could give more than one answer. E.g. if they kept their horse(s) in more than one type of premises, they could answer with all the premises that applied to them.

The introductory message and full questionnaire are available on:

http://www.surveymonkey.com/s/horseownersurvey

Survey response

A total of 4187 respondents participated in the survey between the 16th of November 2010 and the 10th of March 2011, 4094 of which provided valid British post-codes. Seventy-nine respondents who failed to provide post-codes and 14 with post-codes from the crown dependencies and outside the UK were excluded (Table 2.1). The websites, media and e-mail addresses through which information about the questionnaire was dispersed had an estimated audience of 400,000 people. However, the size of the denominator population could not be calculated as the online nature of the questionnaire meant that there was unrestricted access

and there could be overlap between the audiences of different websites, media and e-mail addresses.

When the data from the included and excluded respondents were compared, more excluded respondents were found to keep horses in livestock farms (P=0.03, OR=3, 95% CI 2.24-7.8) and racing yards (P<0.001, OR=15, 95% CI 3.34-67.91). Also, there were no respondents between the ages of 45 and 54 years amongst the 11 excluded respondents who answered this question, compared to 919/3282 (28%) of included respondents.

From this point, only results relating to the 4094 respondents with valid British post-codes will be reported.

Table 2.1: Breakdown of included and excluded respondents who participated in the questionnaire between the 16th of November 2010 and the 10th of March 2011. (GB = Great Britain, PC = post code).

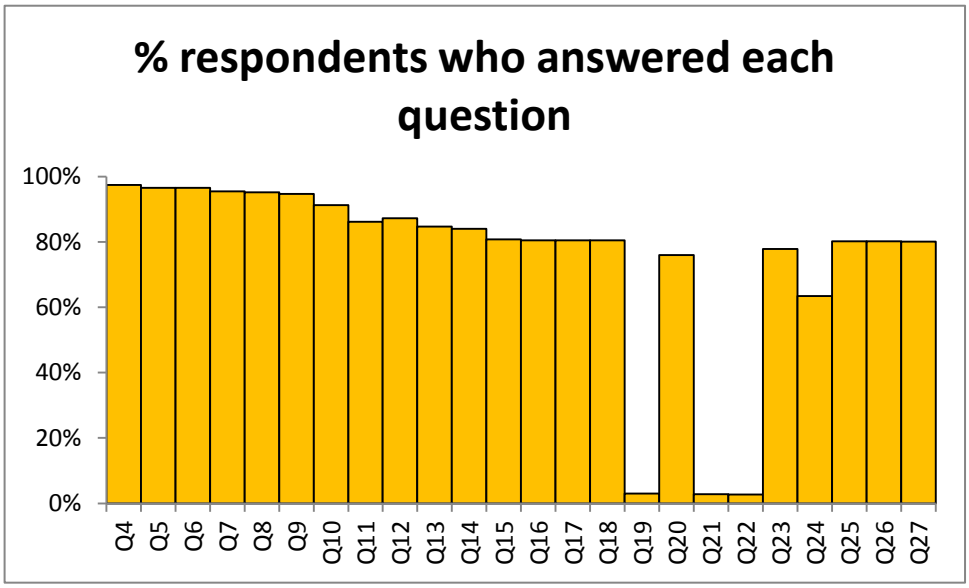
Category	Number of respondents (%)	Horses associated with respondents (%)
Valid GB PC	4094 (97.77%)	16676 (99.56%)
No PC/invalid PC	79 (1.89%)	31 (0.19%)
Belfast	5 (0.07%)	42 (0.25%)
Guernsey	3 (0.07%)	5 (0.03%)
Jersey	3 (0.07%)	10 (0.06%)
Isle of Man	2 (0.05%)	5 (0.03%)
Denmark	1 (0.02%)	4 (0.02%)
Total	4187 (100%)	16750 (100%)

2.2.2 Response rates to individual questions

After the initial questions about post-code and address, 3988/4094 (97%) of respondents answered the initial mandatory question and 3277/4094 (80%) answered the final mandatory question in the questionnaire (excluding a separate section about the National Equine Database which had a 72% response rate). Non mandatory questions relating to international travel, countries that horses were imported from and reasons for importing shared a response rate of 3%, with 112, 115 and 112 respondents out of 4094 answering these questions respectively. For the non-mandatory questions relating to vaccination, 3188/4094 (78%) respondents answered and 2598/4094 answered a question about membership of equestrian societies. For the purposes of analysis of vaccination practice, true "missing" respondents'

answers were those which were also "missing" for the next mandatory question on the questionnaire. Respondents who omitted to answer the question about vaccination but answered the next mandatory question taken to have given a negative response to the vaccination questions. This subset accounted for 97 of 318, 160 and 2984 negative answers for influenza, tetanus and herpes vaccination respectively and raised the total number of responses to the vaccination questions to 3285.

Figure 2.1: The percentage of respondents with valid British postcodes who answered each question in the questionnaire. Questions 1-3 related to the respondents' post-codes and addresses. Questions 28-30 related to the National Equine Database (outside the remit of this study.) Questions 19, 21 and 22 were non-mandatory questions relating to international travel, countries that horses were imported from and reasons for importing. Of the small number respondents (121 and 112 respectively) who answered that they travelled internationally with their horses and imported horses, 100% answered questions 19 and 21 relating to these activities. Questions 23 and 24 were non-mandatory questions relating to vaccination and membership of equestrian societies (respectively).



2.2.3 Data analysis

2.2.3.1 Descriptive summary

Only respondents who listed valid British post-codes were included in the main analysis. A descriptive summary of the responses to the questionnaire was produced. This included post code area and region of the respondent, numbers of horses associated with each respondent, details about where the horses were kept, passport issuing organizations, the respondents connection to the horses (e.g., owner rider, owner non-rider, professional etc), frequency and

distance horses were travelled for 21 possible equestrian activities, transport practices, international travel, imported horses, vaccination practices, membership of equestrian bodies, and owner sex and age. Descriptive summaries were also made of answers (number of horses owned, type of premises and respondent age and gender) in the questionnaires from the excluded respondents. These were compared using Mann-Whitney and Chi-squared tests (for continuous and categorical data respectively) to check for selection bias.

2.2.3.2 Univariable analysis of the association between variables and vaccination practices

Univariable binary logistic regression was used to test the association between demographic variables and influenza, tetanus and herpes vaccination. The categorical variables tested were type of premises, region, participitation in and travel to activities, the respondent's type of equestrian involvement, the area nets to the premises, international travel, importation and the age-group and gender of the respondent. The association between vaccination and the continuous variables number of horses owned, number of horses on the premises and maximum hours travelled to horse care, local and national events was also tested. Variables with likelihood ratio test P values ≤ 0.05 were considered to be statistically significant. Summary Figures illustrating variables associated with increased and reduced odds of the different types of vaccination were created showing the possible confounding associations between the variables. The statistical packages Minitab version 16 and WINPEPI Compare2, version 1.45 (Abramson 2004) were used for analyses.

2.3 Results

2.3.1 Sample characteristics

2.3.1.1 Respondent demographics

The highest number of respondents (667/4094, 16%) listed South West England post-codes. Fewest respondents (16/4094, 0.3%) listed London post-codes (Table 2.2).

Table 2.2: Numbers and percentages of respondents who listed post-codes from each region and numbers and percentages of horses associated with these respondents.

Region	Respondents (%)	Horses (%)
South West England	667 (16.3)	2634 (15.8)
East Anglia	525 (12.8)	1928 (11.6)
South Central England	492 (12)	2156 (12.9)
Midlands	480 (11.7)	1906 (11.4)
North West England	462 (11.3)	1974 (11.8)
North East England	451 (11)	1930 (11.6)
Scotland	427 (10.4)	1751 (10.5)
South East England	358 (8.7)	1281 (7.7)
Wales	216 (5.3)	1051 (6.3)
London	16 (0.4)	65 (0.4)
Total	4094	16676

The majority of respondents (3127/3284, 95%) were female and 70.2% were over the age of 34. Numbers for each age group are listed on tables 2.7-2.9 with the vaccination analyses. Respondents over the age of 45 years owned significantly more horses than respondents under this age ($P<0.001$). The majority of respondents (3179/4497, 71% answers given) were horse owners that also rode (tables 2.7-2.9).

2.3.1.2 Demographics of horses associated with respondents

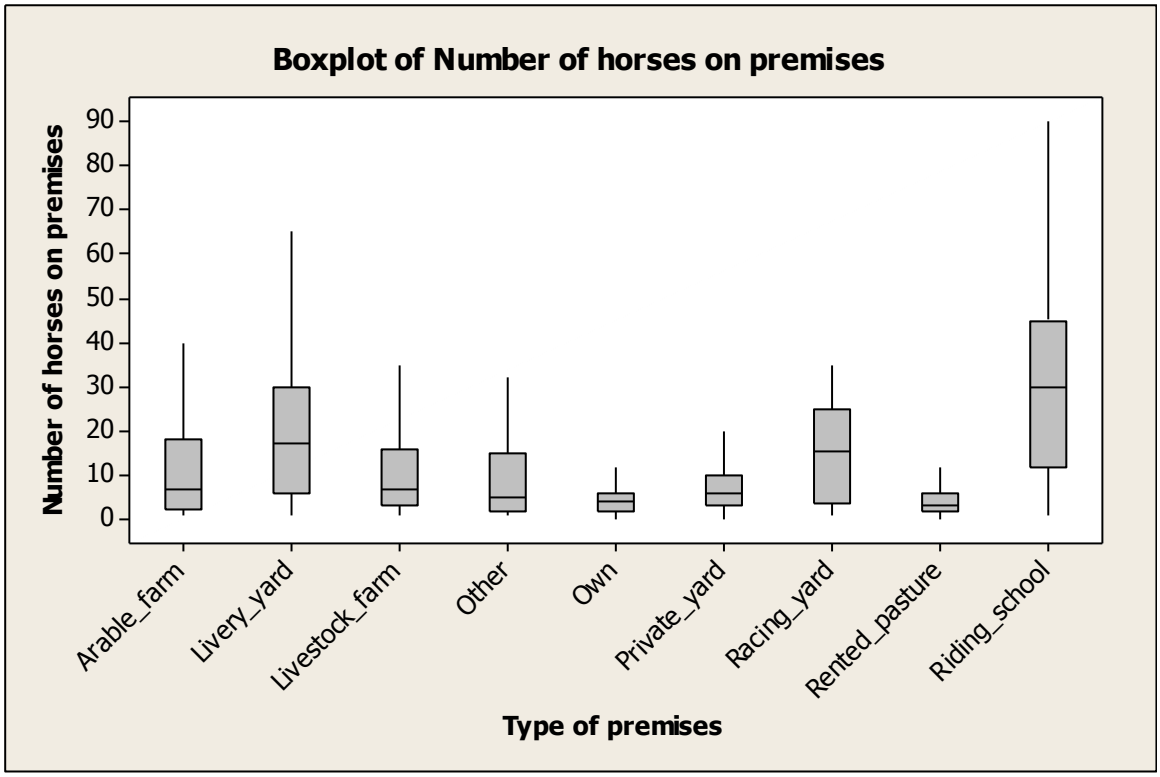
Most respondents listed their own premises as where they kept their horse. Fewest listed a racing yard (Table 2.3)**.**

Table 2.3: Types of premises where horses were kept. Some respondents gave more than one answer to this question, resulting in more answers (4895) than respondents (3951).

Type of premises	Respondents (%)	Horses (%)
Own premises	1275 (26.3)	7256 (31.6)
Livery yard	1252 (25.8)	4328 (18.8)
Private yard	842 (17.4)	3116 (13.6)
Livestock farm	496 (10.2)	2623 (11.4)
Riding school	184 (3.8)	1652 (7.2)
Rented pasture	358 (7.4)	1525 (6.6)
Other premises	201 (4.1)	1423 (6.2)
Arable farm	218 (4.5)	888 (3.9)
Racing yard	19 (0.4)	159 (0.7)
Total answers/horses associated with answers	**4845**	**22970**

There were most horses per premises in riding schools (median 30, interquartile range (IQR) 12-45) and fewest per premises at rented pasture (median 2, IQR 2-6). (Figure 2.2). Only 19/3826 (0.5%) and 201/3876 (5.1%) of respondents stabled their horses entirely in summer and winter respectively, with 1916/3826 (49.4%) of respondents keeping their horses entirely outdoors in the summer. The majority (3205/4357, 73.5%) of respondents kept their horses 2.5 miles or less from where they lived themselves.

Figure 2.2: Box plot showing median values and interquartile ranges for numbers of horses on different tyupes of premises. Whiskers represent upper and lower limits (maximum 1.5 inter-quartile range above and below quartiles)



Of 21 possible activities, most respondents (93.5%) claimed to participate in hacking or leisure riding. The numbers of respondents claiming to participate in each of the 21 activities are listed in Table 2.4. Respondents travelled a median of 1 hour for local events and horse care and a median of 2 hours to national events (Table 2.5). Most respondents (2166/4019, 53.9%) transported their own horse in their own vehicle. However, 624/4019 (15.5%) of respondents transported their horses mixed with horses from different premises. Of 3297 respondents, 1401 (42.5%) stated that their horses had spent nights away from home in the past year. Only 121/3295 (3.7%) respondents claimed to travel internationally with their horses and 112/3188 (3.5%) claimed to import horses. Most respondents who claimed to import (51/112, 45.5%) listed Ireland as the source of their imported horses. Germany, France, the Netherlands and Belgium were the next most listed countries for importation with 17 (15.2%), 15 (13.4%) , 13 (11.6%) and 11(9.8%) respondents (respectively) listing these countries as the origins for their imported horses.

Table 2.4: Numbers of respondents who participate in 21 possible activities.

Activity	Respondents who participate in activity	% Total respondents
Hacking	3240	93.5%
Farrier	3153	91.0%
Riding lessons	2253	65.0%
Unaffiliated events	1973	56.9%
Training	1930	55.7%
Using facilities at a different premises	1885	54.4%
Dressage	1791	51.7%
Showing	1403	40.5%
Show-jumping	1395	40.2%
Riding/pony club	1392	40.2%
Affiliated events	1043	30.1%
Hunting	807	23.3%
Eventing	773	22.3%
Endurance	633	18.3%
Breeding	598	17.3%
Other	518	14.9%
Trailblazers	384	11.1%
Driving	254	7.3%
Western	163	4.7%
Point-to-point	104	3.0%
Vaulting	49	1.4%
Total respondents	**3466**	**100.0%**

Table 2.5: Descriptive statistics for the time spent travelling for local and national events and for horse care (veterinary and farrier attention) by 3439 respondents who answered this question.

Purpose of travel	Total hours travel	Mean hours travel	Lower quartile	Median hours travel	Upper quartile
Local events	6586	1.915	1	1	2
National events	13157	3.826	0	2	4
Horse Care	7875	2.29	1	1	2

2.3.2 Vaccination

2.3.2.1 Descriptive statistics

Of 3285 respondents, 3174 (96.6%) vaccinated their horses against at least one of influenza, tetanus and herpes virus. In addition, 3 respondents vaccinated against West Nile Disease, 3 against Equine Viral Arteritis, 2 against rabies and 1 against botulism. A further 4 respondents believed that mite treatment, vitamin B supplementation and herbal remedies constituted vaccination. Although there was a question regarding strangles vaccination in the questionnaire, this was not analysed due as the strangles vaccine was withdrawn from the market between 2006 and 2011. The numbers of respondents who vaccinated against influenza, tetanus and herpes virus are summarised on Table 2.6.

Table 2.6: Numbers and percentages of respondents who vaccinated their horses against influenza, tetanus and herpes virus.

Type of vaccination	Respondents who vaccinate (%)	Respondents who don't vaccinate (%)
Influenza	2967 (90.3)	318 (9.7)
Tetanus	3125 (95.1)	160 (4.9)
Herpes virus	301 (9.2)	2984 (90.8)
Vaccination (any of above)	3174 (96.6)	111 (3.4)

2.3.2.2 Univariable analysis of factors associated with vaccination

The results of univariable analyses into the associations between reported influenza, tetanus, herpes and any vaccination and region, the type of premises where horses were kept, engagement in and travel to any activity, type of activity, type of area adjacent to the premises where the respondent keeps their horse(s), international travel, importation, the age and gender of the respondent, the number of horses owners, the number of horses on the same premises and the maximum hours travelled to equestrian events are listed in tables 2.7-2.9 and summarised in Figures 2.3-2.9. A further table and diagram describing the association between demographic variables and any vaccination are listed in the appendix (Table A1 and Figures A.2 and A.3). The associations between the separate demographic variables that are associated with vaccination are also described (with odds ratios) in the appendix (Table. A.2).

Table 2.7: Description of the proportions of horses vaccinated and unvaccinated against equine influenza associated with demographic variables, including univariable odds ratios and likelihood ratio test P values of association with influenza vaccination. Blue and yellow highlighting denote decreased and increased odds (respectively) of a variable being associated with influenza vaccination.

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against influenza (%)	Vaccinated against influenza (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Type of premises									
Arable farm	182	12 (6.6)	170 (93.4)	0.44	0.30	0.15	0.127	1.55	0.85-2.82
Livery yard	1040	51 (4.9)	989 (95.1)	0.96	0.16	<0.001	<0.001	2.62	1.92-3.57
Livestock farm	416	51 (12.3)	365 (87.7)	-0.31	0.16	0.058	0.065	0.73	0.53-1.01
Other premises	170	16 (9.4)	154 (90.6)	0.03	0.27	0.903	0.903	1.03	0.61-1.75
Own premises	1072	141 (13.2)	931 (86.8)	-0.56	0.12	<0.001	<0.001	0.57	0.45-0.73
Private yard	696	52 (7.5)	644 (92.5)	0.35	0.16	0.027	0.022	1.42	1.04-1.93
Racing yard	14	1 (7.1)	13 (92.9)	0.33	1.04	0.749	0.737	1.4	0.18-10.7
Rented pasture	315	47 (14.9)	268 (85.1)	-0.56	0.17	0.001	0.002	0.57	0.41-0.8
Riding school	138	14 (10.1)	124 (89.9)	-0.05	0.29	0.85	0.851	0.95	0.54-1.67
Region									
Midlands	372	23 (6.2)	349 (93.8)				0.01	1(REF)	
South Central England	385	25 (6.5)	360 (93.5)	-0.05	0.30	0.861	0.861	0.95	0.53-1.7
South East England	304	21 (6.9)	283 (93.1)	-0.12	0.31	0.704	0.704	0.89	0.48-1.64
East Anglia	427	30 (7)	397 (93)	-0.14	0.29	0.633	0.632	0.87	0.5-1.53
North West England	371	33 (8.9)	338 (91.1)	-0.39	0.28	0.164	0.16	0.68	0.39-1.17
London	11	1 (9.1)	10 (90.9)	-0.42	1.07	0.697	0.82	0.66	0.08-5.37
Scotland	347	34 (9.8)	313 (90.2)	-0.50	0.28	0.075	0.073	0.61	0.35 -1.05
North East England	367	38 (10.4)	329 (89.6)	-0.56	0.28	0.041	0.038	0.57	0.33-0.98
South West England	527	77 (14.6)	450 (85.4)	-0.95	0.25	<0.001	<0.001	0.39	0.24-0.63
Wales	174	36 (20.7)	138 (79.3)	-1.38	0.29	<0.001	<0.001	0.25	0.14-0.44
Activities									
Participates in at least 1 activity	3265	313 (9.6)	2952 (90.4)						
No activities	20	5 (25)	15 (75)	-1.15	0.52	0.03	0.05	0.32	0.11-0.88
Travels to activities	2860	223(7.8)	2637(92.2)						
Does not travel	425	95(22.4)	-77.6	-1.23	0.14	0.00	0.00	0.29	0.23-0.38
Affilliated competitions	999	35 (4)	964 (96)	1.35879	0.183416	<0.001	<0.001	3.89	2.72-5.57
Unaffilliated competitions	1870	98 (5)	1772 (95)	1.20262	0.127084	<0.001	<0.001	3.33	2.59-4.27
Hacking	3069	263 (9)	2806 (91)	1.29329	0.168972	<0.001	<0.001	3.64	2.62-5.08
Hunting	756	45 (6)	711 (94)	0.648133	0.166538	<0.001	<0.001	1.91	1.38-2.65
Breeding	569	63 (11)	506 (89)	-0.183658	0.14892	0.217	0.224	0.83	0.62-1.11

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against influenza (%)	Vaccinated against influenza (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Dressage	2699	69 (3)	2630 (97)	1.9599	0.140132	<0.001	<0.001	7.1	5.39-9.34
Trailblazers	359	16 (4)	343 (96)	0.903114	0.262884	0.001	<0.001	2.47	1.47-4.13
Show jumping	1314	63 (5)	1251 (95)	1.08208	0.145522	<0.001	<0.001	2.95	2.22-3.92
Eventing	728	25 (3)	703 (97)	1.29177	0.212784	<0.001	<0.001	3.64	2.4-5.52
Endurance	604	36 (6)	568 (94)	0.617702	0.183033	0.001	<0.001	1.85	1.3-2.65
Driving	239	33 (14)	206 (86)	-0.43949	0.197556	0.026	0.033	0.64	0.44-0.95
Western	156	19 (12)	137 (88)	-0.272046	0.252247	0.281	0.296	0.76	0.46-1.25
Showing	1326	99 (7)	1227 (93)	0.444639	0.126716	<0.001	<0.001	1.56	1.22-2
vaulting	44	2 (5)	42 (95)	0.819215	0.726165	0.259	0.2	2.27	0.55-9.42
Point-to-point	97	6 (6)	91 (94)	0.497948	0.425685	0.242	0.209	1.65	0.71-3.79
Riding	1318	56 (4)	1262 (96)	1.24213	0.151832	<0.001	<0.001	3.46	2.57-4.66
Lessons	2134	110 (5)	2024 (95)	1.40082	0.124307	<0.001	<0.001	4.06	3.18-5.18
Farrrier	2992	272 (9)	2720 (91)	0.621838	0.172718	<0.001	0.001	1.86	1.33-2.61
Facilities	1785	83 (5)	1702 (95)	1.33748	0.132972	<0.001	<0.001	3.81	2.94-4.94
lessons	1835	97 (5)	1738 (95)	1.16999	0.127369	<0.001	<0.001	3.22	2.51-4.14
Other activities	496	45 (9)	451 (91)	0.083851	0.168819	0.619	0.617	1.09	0.78-1.51
Respondents' type of equestrian involvement									
Breeder	269	44 (16.4)	225 (83.6)	-0.67	0.18	<0.001	<0.001	0.51	0.36-0.72
Donkey owner	41	10 (24.4)	31 (75.6)	-1.12	0.37	0.002	0.006	0.33	0.16-0.67
Livery yard proprietor	103	9 (8.7)	94 (91.3)	0.12	0.35	0.743	0.739	1.12	0.56-2.25
Member travelling community	3	0	3 (100)	20.16	25553.20	0.999	nc	∞	0-∞
Non-owner that loans/rides	80	13 (16.3)	67 (83.8)	-0.61	0.31	0.047	0.063	0.54	0.3-0.99
Other	490	55 (11.2)	435 (88.8)	-0.33	0.24	0.176	0.192	0.72	0.45-1.16
Owner non rider	220	50 (22.7)	170 (77.3)	-1.12	0.17	<0.001	<0.001	0.33	0.23-0.46
Owner that rides	2940	251 (8.5)	2689 (91.5)	0.95	0.15	<0.001	<0.001	2.58	1.92-3.47
Professional	298	17 (5.7)	281 (94.3)	0.62	0.26	0.016	0.009	1.85	1.12-3.07
Thoroughbred industry employee	25	3(12)	22 (88)	-0.24	0.62	0.694	0.703	0.78	0.23-2.64
Area next to premises									
Coastal	73	10 (13.7)	63 (86.3)	-0.40	0.35	0.243	0.265	0.67	0.34-1.32
Grazing	2770	268 (9.7)	2502 (90.3)	0.00	0.16	0.981	0.981	1	0.73-1.38
Hay	1145	93 (8.1)	1052 (91.9)	0.28	0.13	0.028	0.025	1.33	1.03-1.71
Industrial	69	3 (4.3)	66(95.7)	1.28	0.72	0.076	0.093	3.58	0.87-14.7
Other	490	55 (11.2)	435 (88.8)	-0.20	0.16	0.211	0.219	0.82	0.6-1.12
Running water	765	70 (9.2)	695 (90.8)	0.08	0.14	0.571	0.596	1.08	0.82-1.43
Semirural	728	52 (7.1)	676 (92.9)	0.41	0.16	0.009	0.007	1.51	1.11-2.06
Standing water	383	26 (6.8)	357 (93.2)	0.43	0.21	0.043	0.033	1.54	1.01-2.33
Urban	177	14 (7.9)	163 (92.1)	0.23	0.28	0.414	0.4	1.26	0.72-2.21
Woodland	1283	111 (8.7)	1172 (91.3)	0.20	0.12	0.111	0.108	1.22	0.96-1.55
Missing	198								

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against influenza (%)	Vaccinated against influenza (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
International travel									
No international travel	3165	314 (9.9)	2851 (90.1)					REF 1	
International travel	120	4 (3.3)	116 (96.7)	1.16	0.51	0.023	0.006	3.19	1.17 - 8.31
Imports									
Respondent doesn't import	2998	305 (10.2)	2693(89.8)					REF1	
Respondent imports	112	8 (7.1)	104 (92.9)	0.39	0.37	0.298	0.273	1.47	0.71 - 3.05
Respondent age-group									
16 to 24 years old	404	22 (5.4)	382 (94.6)				0.001	REF 1	
Under 16 years old	46	3 (6.5)	43 (93.5)	-0.19	0.64	0.763	0.441	0.83	0.24-2.87
25 to 34 years old	519	32 (6.2)	487 (93.8)	-0.13	0.29	0.644	0.002	0.88	0.5-1.53
35 to 44 years old	721	65 (9)	656 (91)	-0.54	0.25	0.033	0.491	0.58	0.35-0.96
45 to 54 years old	919	98 (10.7)	821 (89.3)	-0.73	0.24	0.003	0.239	0.48	0.3-0.78
55 years or older	673	98 (14.6)	575 (85.4)	-1.08	0.24	<0.001	<0.001	0.34	0.21-0.55
Respondent gender									
Female	3127	299 (9.6)	2828 (90.4)					REF 1	
Male	158	19	139	-0.26	0.25	0.31	0.31	0.77	0.47-1.27
Horses owned									
Odds change per extra horse owned				-0.02	0.01	0.002	0.003	0.98	0.97-0.99
Horses on premises									
Odds change per extra horse on premises				0.01	0.00	0.004	0.002	1.01	1-1.02
Maximum number of hours travelled									
Odds change per extra hour travelled to horse care				0.00	0.01	0.593	0.607	1	0.98-1.01
Odds change per extra hour travelled to local events				0.05	0.03	0.076	0.014	1.05	0.99-1.12
Odds change per extra hour travelled to national events				0.06	0.02	0.008	<0.001	1.06	1.02-1.11

Table 2.8: Description of the proportions of horses vaccinated and unvaccinated against tetanus associated with demographic variables, including univariable odds ratios and likelihood ratio test P values of association with tetanus vaccination. Blue and yellow highlighting denote decreased and increased odds (respectively) of a variable being associated with tetanus vaccination.

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against tetanus (%)	Vaccinated against tetanus (%)	Coefficient	Standard error of coefficient	Wald P value	Liklihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Type of premises									
Arable farm	182	7 (3.9)	175 (96.1)	0.26	0.39	0.51	0.494	1.3	0.6-2.81
Livery yard	1040	39 (3.8)	1001 (96.2)	0.38	0.19	0.043	0.038	1.46	1.01-2.11
Livestock farm	416	27 (6.5)	389 (93.5)	-0.36	0.22	0.102	0.115	0.7	0.46-1.07
Other premises	170	8 (4.7)	162 (95.3)	0.04	0.37	0.918	0.918	1.04	0.5-2.15
Own premises	1072	46 (4.3)	1026 (95.7)	0.19	0.18	0.283	0.278	1.21	0.85-1.72
Private yard	696	33 (4.7)	663 (95.3)	0.04	0.20	0.858	0.858	1.04	0.7-1.53
Racing yard	14	1 (7.1)	13 (92.9)	-0.41	1.04	0.694	0.71	0.66	0.09-5.11
Rented pasture only	315	22 (7)	293 (93)	-0.43	0.24	0.069	0.082	0.65	0.41-1.03
Riding school	138	11 (8)	127 (92)	-0.56	0.33	0.088	0.11	0.57	0.3-1.09
Missing	143								
Region									
Midlands	372	12 (3.2)	360 (96.8)					REF 1	
East Anglia	427	21 (4.9)	406 (95.1)	-0.44	0.37	0.227	0.227	0.64	0.31-1.33
London	11	1 (9.1)	10 (90.9)	-1.10	1.09	0.375	0.365	0.33	0.04-2.82
North East England	367	14 (3.8)	353 (96.2)	-0.17	0.40	0.664	0.664	0.84	0.38-1.84
North West England	371	22 (5.9)	349 (94.1)	-0.64	0.37	0.082	0.076	0.53	0.26-1.08
Scotland	347	24 (6.9)	323 (93.1)	-0.80	0.36	0.027	0.022	0.45	0.22-0.91
South Central England	385	17 (4.4)	368 (95.6)	-0.33	0.38	0.396	0.393	0.72	0.34-1.53
South East England	304	12 (3.9)	292 (96.1)	-0.21	0.42	0.615	0.615	0.81	0.36-1.83
South West England	527	25 (4.7)	502 (95.3)	-0.40	0.36	0.262	0.253	0.67	0.33-1.35
Wales	174	12 (6.9)	162 (93.1)	-0.80	0.42	0.057	0.059	0.45	0.2-1.02
Activities									
Participates in at least 1 activity	3265	159 (4.9)	3106 (95.1)						
No activities	20	1(5)	19 (95)	-0.03	1.03	0.98	0.98	0.97	0.13-7.31
Travels to activities	2860	123(4.3)	2737(95.7)						
Does not travel	425	37(8.7)	388(91.3)	-0.75	0.20	0.00	0.00	0.47	0.32-0.69
Affilliated competitions	999	25 (3)	974 (97)	0.894122	0.221131	<0.001	<0.001	2.45	1.59-3.77
Unaffilliated competitions	1870	57 (3)	1813 (97)	0.915108	0.169015	<0.001	<0.001	2.5	1.79-3.48
Hacking	3069	133 (4)	2936 (96)	1.14854	0.224025	<0.001	<0.001	3.15	2.03-4.89
Hunting	756	27 (4)	729 (96)	0.40463	0.215279	0.06	0.05	1.5	0.98-2.29
Breeding	569	22 (4)	547 (96)	0.282406	0.234341	0.228	0.208	1.33	0.84-2.1
Dressage	1699	48 (3)	1651 (97)	0.960699	0.176197	<0.001	<0.001	2.61	1.85-3.69
Trailblazers	359	13 (4)	346 (96)	0.342076	0.294917	0.246	0.225	1.41	0.79-2.51

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against tetanus (%)	Vaccinated against tetanus (%)	Coefficient	Standard error of coefficient	Wald P value	Liklihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Show jumping	1314	48 (4)	1266 (96)	0.463121	0.176323	0.009	0.007	1.59	1.12-2.25
Eventing	728	21 (3)	707 (97)	0.660286	0.237993	0.006	0.003	1.94	1.21-3.09
Endurance	604	15 (2)	589 (98)	0.808766	0.275055	0.003	0.001	2.25	1.31-3.85
Driving	239	10 (4)	229 (96)	0.170686	0.333735	0.609	0.601	1.19	0.62-2.28
Western	156	10 (6)	146 (94)	-0.307686	0.337419	0.362	0.381	0.74	0.38-1.42
Showing	1326	41 (3)	1285 (97)	0.706545	0.184703	<0.001	<0.001	2.03	1.41-2.91
vaulting	44	1 (2)	43 (98)	0.79677	1.01482	0.432	0.371	2.22	0.3-16.21
Point-to-point	97	5 (5)	92 (95)	-0.0615317	0.46653	0.895	0.896	0.94	0.38-2.35
Riding	1318	35 (3)	1283 (97)	0.911315	0.19466	<0.001	<0.001	2.49	1.7-3.64
Lessons	2134	77 (4)	2057 (96)	0.730496	0.162658	<0.001	<0.001	2.08	1.51-2.86
Farrrier	2992	139 (5)	2853 (95)	0.460373	0.24257	0.058	0.056	1.58	0.99-2.55
Facilities	1785	51 (3)	1734 (97)	0.97993	0.173428	<0.001	<0.001	2.66	1.9-3.74
lessons	1835	57 (3)	1778 (97)	0.869287	0.168989	<0.001	<0.001	2.39	1.71-3.32
Other activities	496	14 (3)	482 (97)	0.642823	0.284129	0.024	0.014	1.9	1.09-3.32
Respondents' type of equestrian involvement									
Breeder	269	17 (6.3)	252 (93.7)	-0.30	0.26	0.251	0.268	0.74	0.44-1.24
Donkey owner	41	3 (7.3)	38 (92.7)	-0.44	0.61	0.468	0.494	0.64	0.2-2.11
Livery yard proprietor	103	6 (5.8)	97 (94.2)	-0.20	0.43	0.648	0.657	0.82	0.35-1.91
Member travelling community	3	0	3 (100)	20.08	35466.70	1	nc	∞	0-∞
Non-owner that loans/rides	80	11 (13.8)	69 (86.3)	-1.18	0.34	0	0.002	0.31	0.16-0.59
Other	490	22 (4.5)	468 (95.5)	0.32	0.42	0.452	0.431	1.38	0.6-3.16
Owner non rider	220	19 (8.6)	201 (91.4)	-0.67	0.26	0.008	0.014	0.51	0.31-0.84
Owner that rides	2940	125 (4.3)	2815 (95.7)	0.93	0.20	<0.001	<0.001	2.54	1.72-3.77
Professional	298	6 (2)	292 (98)	0.97	0.42	0.021	0.0007	2.65	1.16-6.03
Thoroughbred industry employee	25	1 (4)	24 (96)	0.21	1.02	0.839	0.834	1.23	0.17-9.15
Area next to premises									
Coastal	73	7 (9.6)	66 (90.4)	-0.75	0.41	0.064	0.091	0.47	0.21-1.05
Grazing	2770	129 (4.7)	2641 (95.3)	0.27	0.21	0.188	0.2	1.31	0.88-1.96
Hay	1145	52 (4.5)	1093 (95.5)	0.11	0.17	0.522	0.519	1.12	0.8-1.57
Industrial	69	3 (4.3)	66 (95.7)	0.12	0.60	0.839	0.836	1.13	0.35-3.63
Other	490	22 (4.5)	468 (95.5)	0.10	0.23	0.671	0.668	1.1	0.7-1.75
Running water	765	33 (4.3)	732 (95.7)	0.16	0.20	0.414	0.408	1.18	0.8-1.74
Semirural	728	34 (4.7)	694 (95.3)	0.06	0.20	0.776	0.775	1.06	0.72-1.56
Standing water	383	14 (3.7)	369 (96.3)	0.33	0.29	0.242	0.222	1.4	0.8-2.44
Urban	177	9 (5.1)	168 (94.9)	-0.05	0.35	0.892	0.892	0.95	0.48-1.9
Woodland	1283	45 (3.5)	1238 (96.5)	0.52	0.18	0.004	0.003	1.68	1.18-2.38
International travel									
No international travel	3165	158 (5)	3007 (95)					REF 1	
International travel	120	2 (1.7)	118 (98.3)	1.13	0.72	0.115	0.057	3.1	0.76 - 12.66

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against tetanus (%)	Vaccinated against tetanus (%)	Coefficient	Standard error of coefficient	Wald P value	Liklihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Imports									
Respondent doesn't import	2998	148 (4.9)	2850 (95.1)					REF 1	
Respondent imports	112	4 (3.6)	108 (96.4)	0.34	0.52	0.513	0.491	1.4	0.51-3.86
Respondent age-group									
45 to 54 years old	919	36 (3.9)	883 (96.1)				0.106	REF 1	
35 to 44 years old	721	33 (4.6)	688 (95.4)	-0.16	0.25	0.509	0.676	0.85	0.52-1.38
25 to 34 years old	519	25 (4.8)	494 (95.2)	-0.22	0.27	0.417	0.951	0.81	0.48-1.36
55 years or older	673	35 (5.2)	638 (94.8)	-0.30	0.24	0.222	0.659	0.74	0.46-1.2
16 to 24 years old	404	26 (6.4)	378 (93.6)	-0.52	0.26	0.048	0.133	0.59	0.35-1
Under 16 years old	46	5 (10.9)	41 (89.1)	-1.10	0.50	0.029	0.098	0.33	0.12-0.9
Respondent gender									
Female	3127	140 (4.5)	2987 (95.5)					REF 1	
Male	158	20 (12.7)	138 (87.3)	-1.13	0.25	<0.001	<0.001	0.32	0.2-0.53
Horses owned									
Odds change per extra horse owned				-0.01	0.01	0.14	0.18	0.99	0.97-1
Horses on premises									
Odds change per extra horse on premises				0.00	0.01	0.93	0.93	1.00	0.99-1.01
Maximum number of hours travelled									
Odds change per extra hour travelled to horse care				-0.01	0.01	0.12	0.10	0.99	0.97-1
Odds change per extra hour travelled to local events				0.07	0.05	0.16	0.04	1.08	0.97-1.19
Odds change per extra hour travelled to national events				0.15	0.04	<0.001	<0.001	1.16	1.08-1.26

Table 2.9: Description of the proportions of horses vaccinated and unvaccinated against equine herpes virus associated with demographic variables, including univariable odds ratios and likelihood ratio test P values of association with influenza vaccination. Blue and yellow highlighting denote decreased and increased odds (respectively) of a variable being associated with tetanus vaccination.

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against herpes (%)	Vaccinated against herpes (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Type of premises									
Arable farm	182	170 (93.4)	12 (6.6)	-4.93	0.31	0.219	0.196	0.69	0.38-1.25
Livery yard	1040	920 (88.5)	120 (11.5)	0.40	0.12	0.001	0.002	1.49	1.17-1.9
Livestock farm	416	390 (93.8)	26 (6.2)	-0.46	0.21	0.029	0.021	0.63	0.41-0.95
Other premises	170	151 (88.8)	19 (11.2)	0.23	0.25	0.351	0.364	1.26	0.77-2.07
Own premises	1072	987 (92)	85 (8)	-0.23	0.13	0.089	0.084	0.8	0.61-1.03
Private yard	696	625 (89.8)	71 (10.2)	0.15	0.14	0.285	0.291	1.17	0.88-1.54
Racing yard	14	12 (85.7)	2 (14.3)	0.50	0.77	0.51	0.535	1.66	0.37-7.44
Rented pasture only	315	289 (91.8)	26 (8.2)	-0.13	0.21	0.557	0.551	0.88	0.58-1.34
Riding school	138	125 (90.6)	13 (9.4)	0.03	0.30	0.915	0.915	1.03	0.58-1.85
Region									
East Anglia	427	375 (87.8)	52 (12.2)					REF 1	
London	11	9 (81.9)	2 (18.2)	0.47	0.80	0.553	0.895	1.6	0.34-7.62
Midlands	372	339 (91.1)	33 (8.9)	-0.35	0.23	0.132	0.129	0.7	0.44-1.11
North East England	367	328 (89.4)	39 (10.6)	-0.15	0.22	0.494	0.493	0.86	0.55-1.33
North West England	371	340 (91.6)	31 (8.4)	-0.42	0.24	0.079	0.076	0.66	0.41-1.05
Scotland	347	329 (94.8)	18 (5.2)	-0.93	0.28	0.001	0.001	0.39	0.23-0.69
South Central England	385	342 (88.8)	43 (11.2)	-0.10	0.22	0.655	0.655	0.91	0.59-1.39
South East England	304	275 (90.5)	29 (9.5)	-0.27	0.24	0.264	0.259	0.76	0.47-1.23
South West England	527	486 (92.2)	41 (7.8)	-0.50	0.22	0.024	0.023	0.61	0.4-0.94
Wales	174	161 (92.5)	13 (7.5)	-0.54	0.32	0.095	0.082	0.58	0.31-1.1
Activities									
Participates in at least 1 activity	3265	2966(90.8)	299(9.2)						
No activities	20	18(90)	2(10)	0.10	0.75	0.90	0.90	1.1	0.25-4.77
Travels to activities	2860	2588(90.5)	272(9.5)						
Does not travel	425	396(93.2)	29(6.82)	-0.36	0.20	0.08	0.07	0.7	0.47-1.04
Affilliated competitions	999	875 (88)	124 (12)	0.523877	0.123819	<0.001	<0.001	1.69	1.32-2.15
Unaffilliated competitions	1870	1671 (89)	199 (11)	0.427224	0.127236	0.001	0.001	1.53	1.19-1.97
Hacking	3069	2783 (91)	286 (9)	0.319962	0.274768	0.244	0.225	1.38	0.8-2.36
Hunting	756	672 (89)	84 (11)	0.286529	0.135771	0.035	0.038	1.33	1.02-1.74
Breeding	569	480 (84)	89 (16)	0.783909	0.135777	<0.001	<0.001	2.19	1.68-2.86

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against herpes (%)	Vaccinated against herpes (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Dressage	1699	1518 (89)	181 (11)	0.376149	0.123285	0.002	0.002	1.46	1.14-1.85
Trailblazers	359	322 (90)	37 (10)	0.14725	0.185192	0.427	0.433	1.16	0.81-1.67
Show jumping	1314	1178 (90)	136 (10)	0.234006	0.121721	0.055	0.055	1.26	1-1.6
Eventing	728	641 (88)	87 (12)	0.39609	0.134737	0.003	0.004	1.49	1.14-1.94
Endurance	604	540 (89)	64 (11)	0.200645	0.148679	0.177	0.184	1.22	0.91-1.64
Driving	239	217 (91)	22 (9)	0.0054523	0.232396	0.981	0.981	1.01	0.64-1.59
Western	156	137 (88)	19 (12)	0.336572	0.252642	0.183	0.199	1.4	0.85-2.3
Showing	1326	1186 (89)	140 (11)	0.276327	0.121463	0.023	0.023	1.32	1.04-1.67
vaulting	44	32 (73)	12 (27)	1.34298	0.344067	<0.001	<0.001	3.83	1.95-7.52
Point-to-point	97	80 (82)	17 (18)	0.776057	0.274206	0.005	0.009	2.17	1.27-3.72
Riding	1318	1197 (91)	121 (9)	0.0035535	0.123349	0.977	0.977	1	0.79-1.28
Lessons	2134	1931 (90)	203 (10)	0.121844	0.128832	0.344	0.432	1.13	0.88-1.45
Farrrier	2992	2711 (91)	281 (9)	1.04017	0.32801	0.002	0.131	2.83	1.49-5.38
Facilities	1785	1619 (91)	166 (9)	0.0360588	0.12158	0.767	0.767	1.04	0.82-1.32
lessons	1865	1699 (91)	166 (9)	-	0.121575	0.684	0.795	0.95	0.75-1.21
Other activities	496	451 (91)	45 (9)	-	0.16953	0.94	0.94	0.99	0.71-1.38
Respondents' type of equestrian involvement									
Breeder	269	229 (85.1)	40 (14.9)	0.61	0.18	0.001	0.002	1.84	1.29-2.64
Donkey owner	41	40 (97.6)	1 (2.4)	-1.41	1.01	0.166	0.079	0.25	0.03-1.79
Livery yard proprietor	103	89 (86.4)	14 (13.6)	0.46	0.29	0.116	0.136	1.59	0.89-2.82
Member travelling community	3	2 (66.7)	1 (33.3)	1.60	1.23	0.191	nc	4.97	0.45-54.97
Non-owner that loans/rides	80	65 (81.3)	15 (18.8)	0.86	0.29	0.003	0.007	2.36	1.33-4.18
Other	490	452 (92.2)	38 (7.8)	0.39	0.24	0.106	0.121	1.48	0.92-2.38
Owner non rider	220	200 (90.9)	20 (9.1)	-0.01	0.24	0.969	0.969	0.99	0.62-1.59
Owner that rides	2940	2679 (91.1)	261 (8.9)	-0.30	0.18	0.099	0.109	0.74	0.52-1.06
Professional	298	263 (88.3)	35 (11.7)	0.31	0.19	0.106	0.118	1.36	0.94-1.98
Thoroughbred industry employee	25	20 (80)	5 (20)	0.92	0.50	0.068	0.097	2.5	0.93-6.72
Missing	523								
Area next to premises									
Coastal	73	63 (86.3)	10 (13.7)	0.47	0.35	0.178	0.202	1.59	0.81-3.14
Grazing	2770	2514 (90.8)	256 (9.2)	0.06	0.17	0.716	0.714	1.06	0.76-1.48
Hay	1145	1039 (90.7)	106 (9.3)	0.02	0.13	0.89	0.891	1.02	0.79-1.3
Industrial	69	60 (87)	9 (13)	0.41	0.36	0.262	0.285	1.5	0.74-3.06
Other	490	452 (92.2)	38 (7.8)	-0.21	0.18	0.242	0.232	0.81	0.57-1.15
Running water	765	701 (91.6)	64 (8.4)	-0.13	0.15	0.383	0.379	0.88	0.66-1.17
Semirural	728	647 (88.9)	81 (11.1)	0.29	0.14	0.038	0.042	1.33	1.02-1.74
Standing water	383	353 (92.2)	30 (7.8)	-0.19	0.20	0.338	0.327	0.83	0.56-1.22
Urban	177	160 (90.4)	17 (9.6)	0.05	0.26	0.834	0.835	1.06	0.63-1.77
Woodland	1283	1169 (91.1)	114 (8.9)	-0.05	0.12	0.659	0.659	0.95	0.74-1.21

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated against herpes (%)	Vaccinated against herpes (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
International travel									
No international travel	3165	2890 (91.3)	275 (8.7)					REF 1	
International travel	120	94 (78.3)	26 (21.7)	1.07	0.23	<0.001	<0.001	2.91	1.85 -4.57
Imports									
Respondent doesn't import	2998	2734 (91.2)	264 (8.8)					REF 1	
Respondent imports	112	93 (83)	19 (17)	0.75	0.26	0.004	0.007	2.12	1.27-3.52
Respondent age-group									
Under 16 years old	46	27 (58.7)	19 (41.3)				<0.001	REF 1	
25 to 34 years old	519	461 (88.8)	58 (11.2)	-1.72	0.33	<0.001	0.091	0.18	0.09-0.34
16 to 24 years old	404	362 (89.4)	42 (10.4)	-1.80	0.34	<0.001	0.367	0.16	0.08-0.32
35 to 44 years old	721	658 (91.3)	63 (8.7)	-1.99	0.33	<0.001	0.653	0.14	0.07-0.26
55 years or older	673	621 (92.3)	52 (7.7)	-2.13	0.33	<0.001	0.14	0.12	0.06-0.23
45 to 54 years old	919	853 (92.8)	66 (7.2)	-2.21	0.33	<0.001	0.012	0.11	0.06-0.21
Respondent gender									
Female	3127	2848 (91.1)	279 (8.9)					REF 1	
Male	158	136 (86.1)	22 (13.9)	0.50	0.24	0.035	0.074	1.65	1.04-2.63
Horses owned									
Odds change per extra horse owned				0.03	0.01	<0.001	<0.001	1.03	1.01-1.04
Horses on premises									
Odds change per extra horse on premises				0.01	0.00	<0.001	<0.001	1.01	1.01-1.02
Maximum number of hours travelled									
Odds change per extra hour travelled to horse care				0.01	0.01	0.062	0.087	1.01	1-1.03
Odds change per extra hour travelled to local events				0.01	0.01	0.443	0.467	1.01	0.99-1.03
Odds change per extra hour travelled to national events				0.00	0.00	0.207	0.025	1	1-1.01

Figure 2.3: The demographic variables which were associated with reduced odds of influenza vaccination (with 95% confidence intervals less than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with 95% confidence intervals for odds ratios greater than 1).

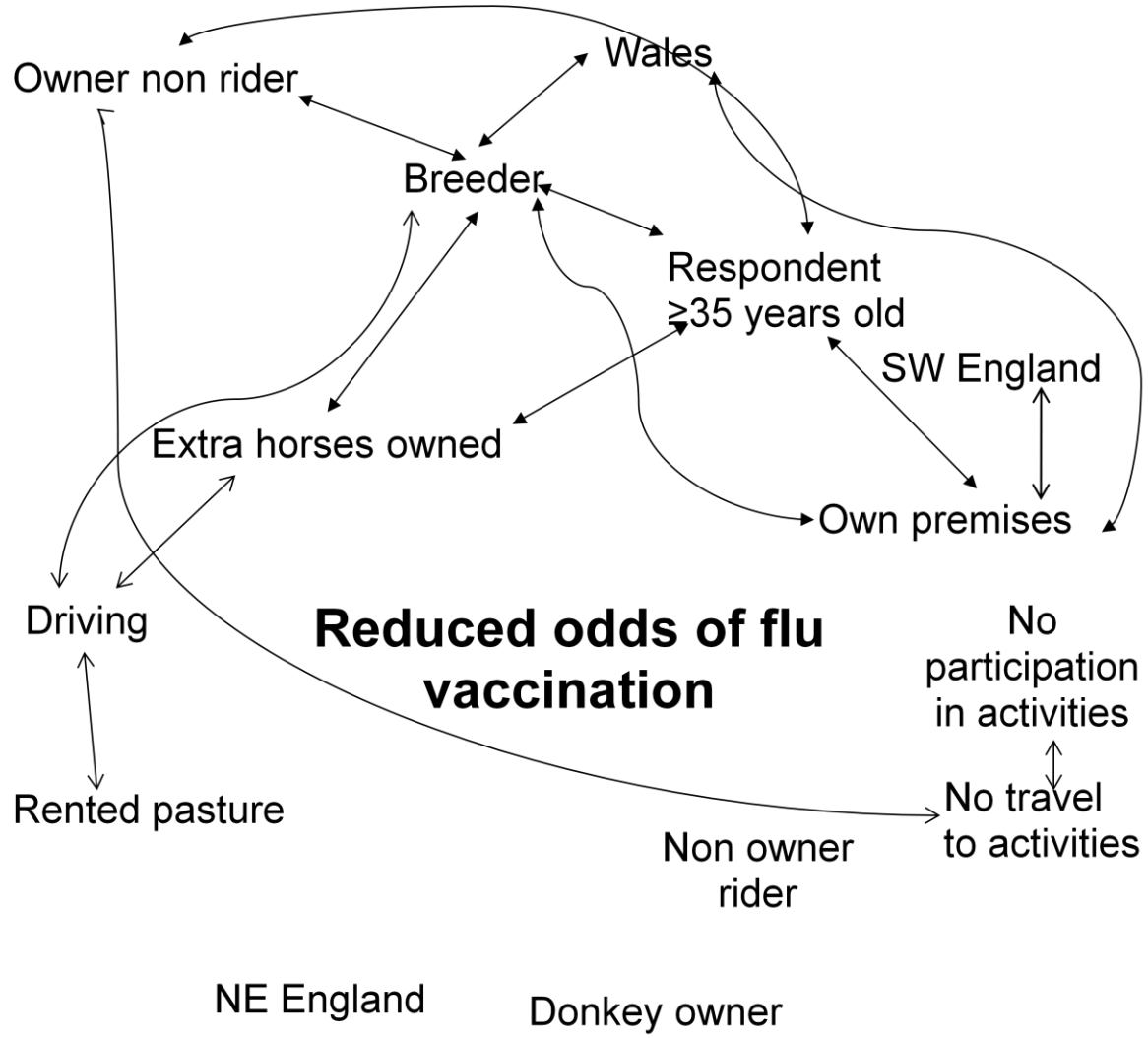


Figure 2.4: The variables which were associated with increased odds of influenza vaccination (with 95% confidence intervals greater than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1). The activities highlighted in brown are those associated with respondents that listed "Riding instructor/coach/professional" as describing their involvement with horses. Affilliated competitive activities were associated with extra hours r=travelled to national events and private yards.

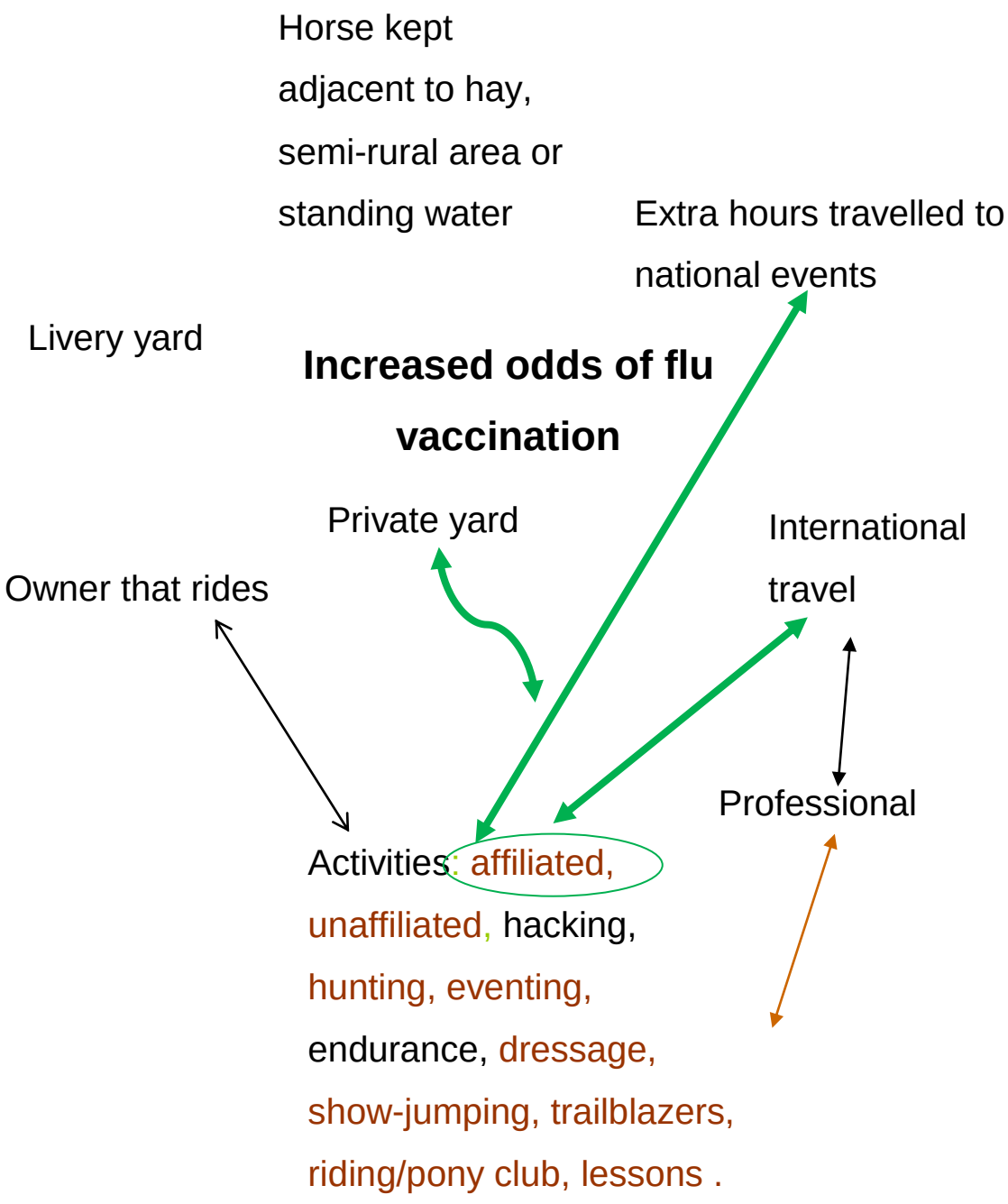


Figure 2.5: The variables which were associated with reduced odds of tetanus vaccination (with 95% confidence intervals less than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1).

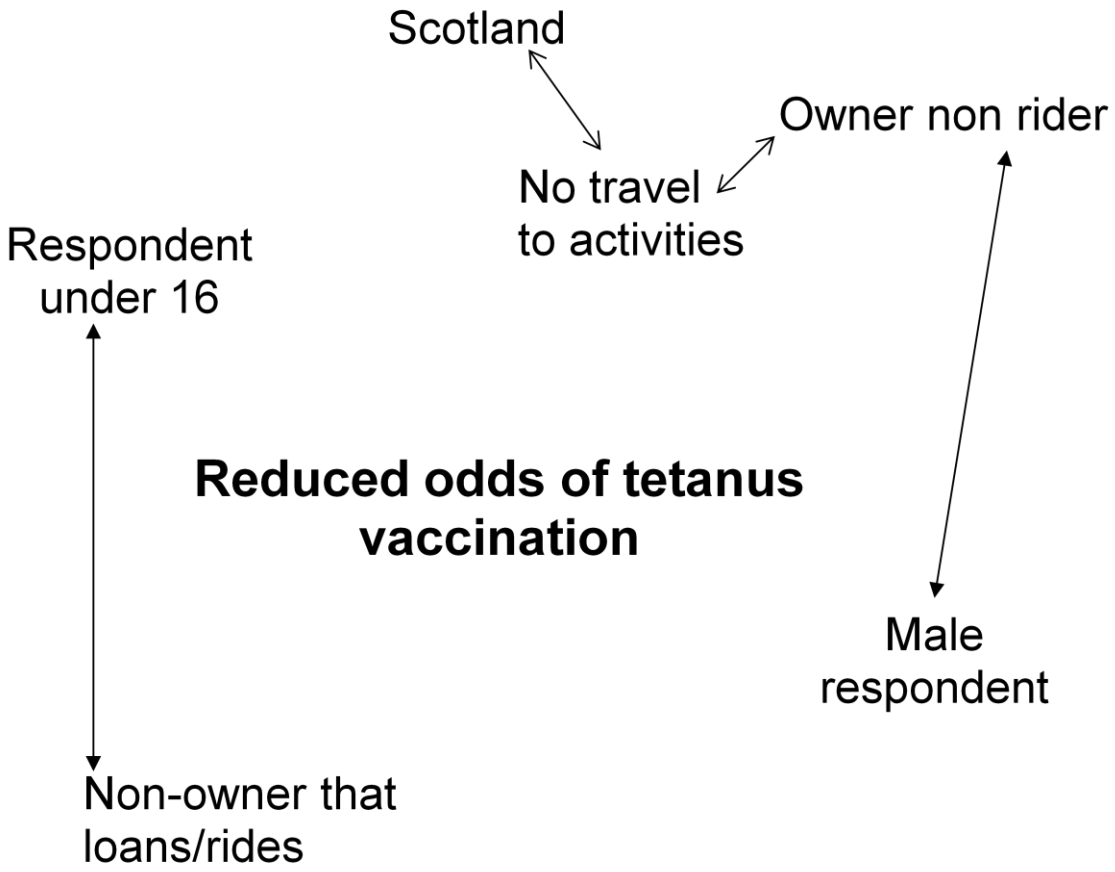


Figure 2.6: The variables which were associated with increased odds of tetanus vaccination (with 95% confidence intervals greater than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1). The activities highlighted in yellow are those associated with respondents that listed "Riding instructor/coach/professional" as describing their involvement with horses.

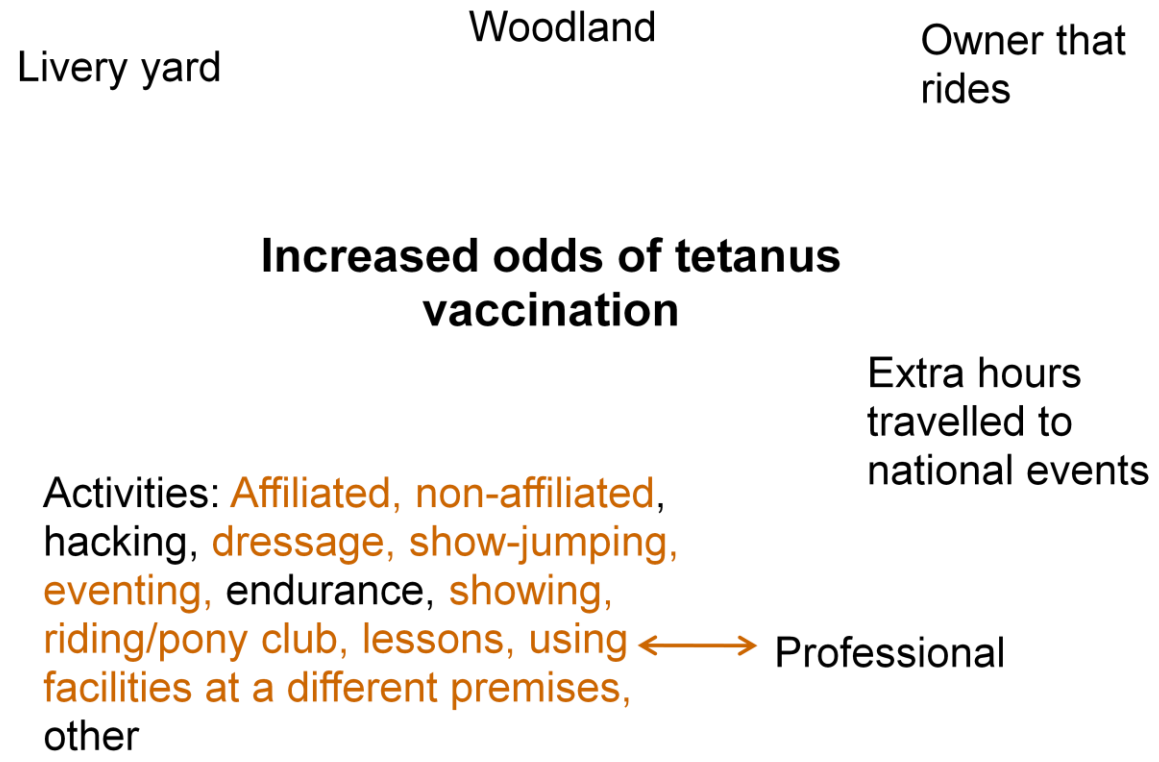


Figure 2.7: The variables which were associated with reduced odds of herpes vaccination (with 95% confidence intervals less than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1).

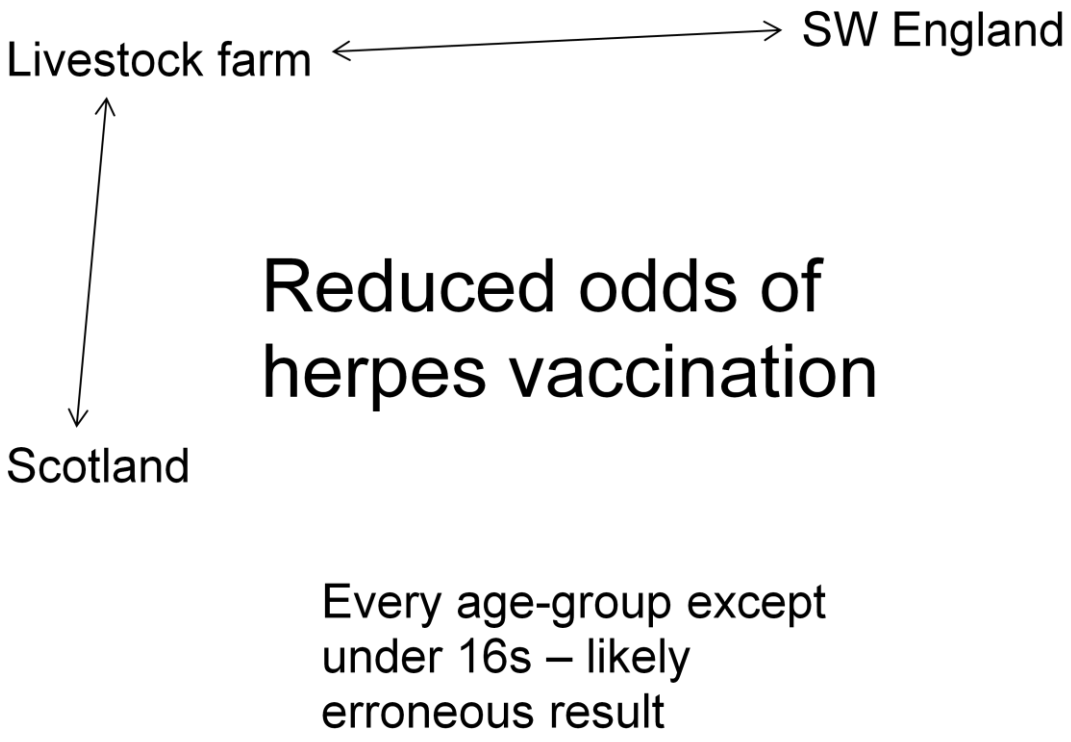
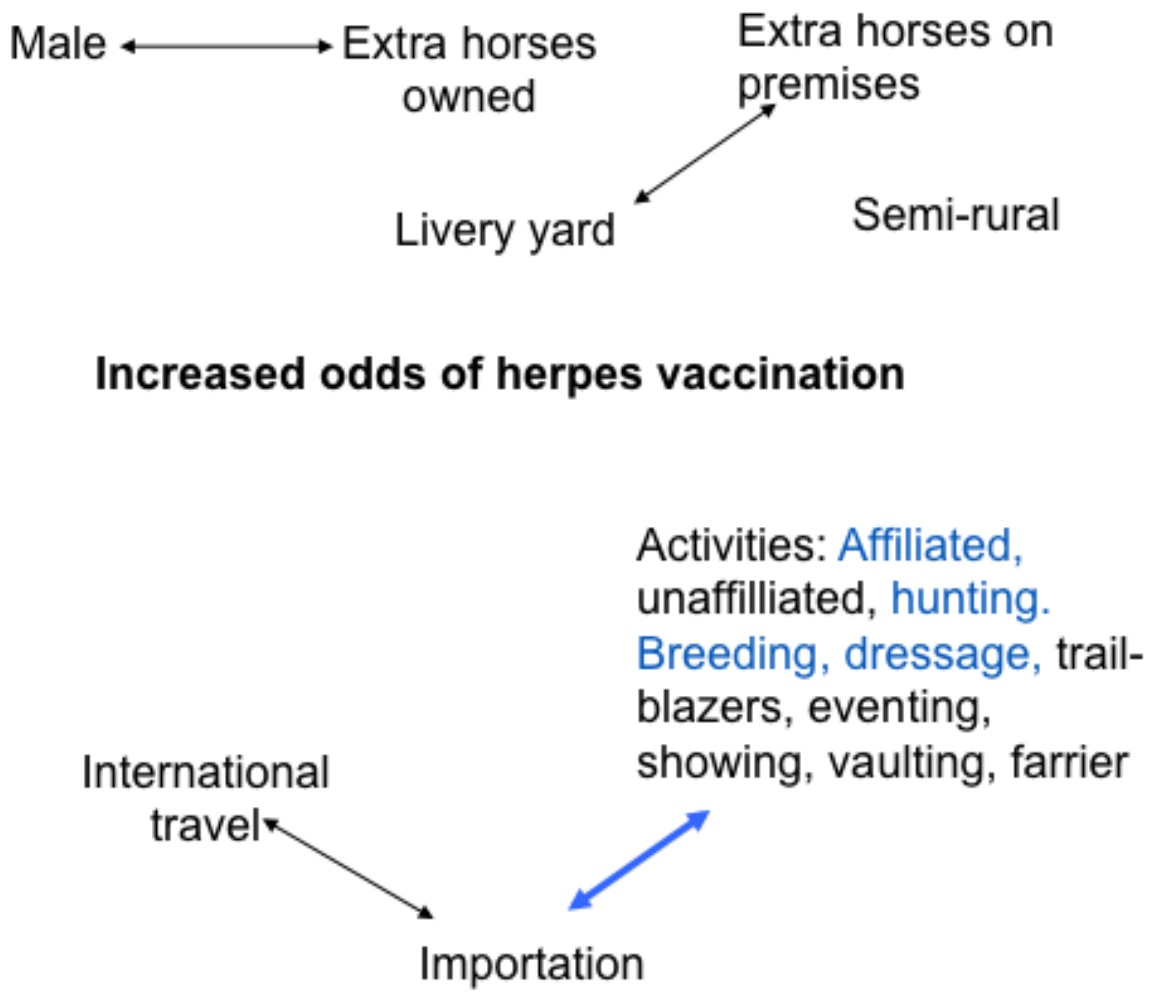


Figure 2.8: The variables which were associated with increased odds of herpes vaccination (with 95% confidence intervals greater than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1). The activities highlighted in yellow are those associated with respondents that listed "Riding instructor/coach/professional professional" as describing their involvement with horses. International travel is associated with affiliated activities. Affiliated activities, hunting, breeding and dressage are associated with importing horses.



2.4 Discussion

2.4.1 Advantages and disadvantages of the web-based format and consideration of bias

In previous studies, horse owners registered with veterinary practices functioned as the sample population for questionnaires relating to equine demographics and management practices (Hotchkiss *et al*., 2007; Ireland *et al*., 2011; Mellor *et al*., 1999). It is likely that owners registered with veterinary practices are more likely to vaccinate their horses that those that are not. Given the primary aim of this study was to investigate the association between demographic variables and vaccination practices, the web-based nature of this present questionnaire was advantageous in avoiding selection bias associated with owners registered with veterinary practices.

According to the Office of National Statistics in 2008, 65% of households in the UK have internet access (Anon, 2008), indicating that the web is a powerful resource for conducting health surveys (Fischbacher *et al*., 2000). The web-based format also facilitated a large sample size and efficient transfer of questionnaire responses to a database for analysis. The broad web audience has enabled the inclusion of respondents from every region in Great Britain and a description of the numbers of respondents from each region. This was not possible in the targeted postal questionnaires of (Hotchkiss *et al*., 2007) and (Mellor *et al*., 1999).

A disadvantage of a web-based format is that the non-response rate cannot be calculated due to the open-access nature of the questionnaire. I.e. it is impossible to know how many horse owners were aware of the questionnaire and chose not to participate in it or to target them retrospectively to investigate non-response bias. A previous questionnaire investigating equine management practices investigated "non-response" bias by comparing the answers of respondents who responded to a first mailing of a postal questionnaire to those who responded to a second mailing and detected minimal differences between them (Hotchkiss *et al*., 2007). In this study, it was possible to compare included respondents (those with valid GB post-codes) and those that were excluded (mostly from the crown dependencies and Ireland) to investigate selection bias. In the excluded population, more respondents kept horses in racing yards and livestock farms, suggesting a difference in the types of equestrian premises in Britain and its neighbours.

It is possible that the horse owners who consider answering a 30 question questionnaire about their horses are those with an interest in equine health and are more likely to vaccinate their horses. This problem is a problem also faced by postal questionnaires but must be kept in mind when interpreting the results.

2.4.2 Over-representation of female respondents

Females made up 95% of questionnaire respondents. This may indicate that there are more female horse owners in Britain or that female horse owners are more likely to participate in questionnaires. A recent web-based survey of cat owners had a similar gender distribution, with 86% of 3122 respondents being female (Habacher *et al*., 2010). Women are reported to be more interested in health related topics on the internet (Fox and Rainie, 2002) and, in a survey of pet-owning households in the USA, women tended to carry the primary responsibility for animal health-care (Wise *et al*., 2003). Interestingly, the male respondents were less likely to vaccinate their horses against tetanus but more likely to vaccinate against herpes virus. The increased likelihood of males vaccinating against herpes virus is likely to be confounded by the increased likelihood of men to be horse breeders (unreported result, OR 2, 95% CI 1.3-3.2) and the common practice of vaccinating broodmares to prevent herpes virus abortion. Ideally, biologically plausible associations highlighted by univariable analyses as being significant (at $P<0.2$) would be analysed in a multivariable regression model to take confounding factors such as the association between gender and equestrian activity into account.

2.4.3 Herpes virus vaccination

Hotchkiss *et al*., (2007) were surprised with their finding that 20.6% of respondents to their questionnaire reported to vaccinate their horses against herpes virus. They expected fewer respondents to vaccinate against herpes as it was conventionally associated with pregnant mares. In that paper, it was suggested that a lack of understanding of what a herpes virus vaccination was led to an erroneously high proportion of positive claims regarding this vaccination. Nineteen of 46 respondents (41%) of respondents under the age of 16 claimed to vaccinate their horses against herpes virus compared to only 9.3% of all 3285 respondents who answered this question. Similarly, 15/80 (19%) of non-owners who rode or loaned horses claimed to vaccinate against herpesvirus. The variables of being aged under 16 and not owning a horse were also associated (OR 4, 95%CI 1.4-11.4). The most likely explanation

for these unexpected results is, that similarly to the what Hotchkiss *et al*., (2007) suspected of their survey respondents, the younger participants in our survey may not have fully understood what herpesvirus vaccination was. The association of East Anglia and point-to-point activities with herpes vaccination could be explained by the outbreak of EHV-1 meningoenecphalopathy in East Anglia and in several national hunt horses over the past few years. Amidst these recent outbreaks, there have been calls for widespread vaccination against herpes virus in order to increase the equine population resistance. Our results indicate that current herpes virus vaccination practices leave much of the British equine population susceptible to this disease. It appears that horses kept on larger premises, with international travel, importation and many activities are more likely to be vaccinated against herpes-virus. However, multivariable logistic regression would be ideally used to decipher which of these factors was primarily associated with herpesvirus vaccination and which were confounded by their associations with other factors. It is difficult to explain why respondents from Scotland and South West England have reduced odds of vaccinating their horses against herpesvirus, especially as respondents from South West England were more likely to breed their horses. This might suggest that owner and veterinary education efforts regarding herpesvirus vaccination should have a regional focus.

2.4.4 Influenza vaccination

There was also regional variation in the case of influenza and tetanus vaccination, with respondents from Wales and South West England being less likely to vaccinate against influenza and respondents from Scotland less likely to vaccinate against Tetanus. This again highlights the regions most vulnerable to disease due to lack of vaccination coverage. It would be useful to compare the prevalence of influenza, tetanus and herpesvirus in these regions.

There is a marked contrast in the influenza vaccination practices between respondents who engage in and travel to activities with their horses and those who do not. (OR 0.3, 95% CI 0.1-0.9, OR 0.3, 95% CI 0.2-0.4 respectively for influenza vaccination by respondents who activities and those who do not engage in any activities with their horse and do not travel away from their own premises.) This can be partially explained by FEI regulations for vaccinating horses at affiliated events as well as the regulations of many national organisations for competitive equestrian pursuits. Despite the common practice of

transporting mares for breeding, respondents who described themselves as breeders or stud owners were also less likely to vaccinate their horses against influenza (OR 0.5, 95%CI 0.4-0.7). Hence (subject to further multivariable analysis of this finding) the movements of unvaccinated horses from the population of this current study for breeding purposes may facilitate dispersal of influenza. As in the case of herpesvirus vaccination, there are many associations between variables (e.g. horse breeding, owning extra horses, being over ≥35 years old, being an owner who doesn't ride.) Multivariable analyses would be necessary to ascertain which of the variables are truly associated with reduced likelihood of influenza vaccination.

The reduced likelihood of donkey owners to vaccinate their animals against influenza (OR0.33, 95% CI 0.16-0.67) is concerning as donkeys are more susceptible to developing severe clinical signs and dying subsequent to influenza (as reviewed by (Thiemann and Bell, 2001). Furthermore, there have been calls for widespread vaccination of donkeys in Britain subsequent to an outbreak of influenza in donkeys in the New Forest (Caerdwell *et al*., 2000). Perhaps it is erroneously thought that "equine" influenza will not affect donkeys.

2.4.5 Tetanus vaccination

Unlike the case of influenza and herpesvirus, travel away from horses' premises is unlikely to have a bearing on tetanus infection. Tetanus is most commonly occurs after an injury involving the skin or hoof of an unvaccinated horse (Green *et al*., 1994). In spite of this, respondents that do not transport their horses to any activity are least likely to vaccinate their horse against tetanus (OR 0.5, 95%CI 0.3-0.7). Furthermore, despite no biologically plausible connection between lack of ridden work and risk of tetanus infection, non-riders who own horses are also less likely to vaccinate their horses against this disease (OR 0.5, 95% CI 0.3-0.8).

2.4.6 Conclusion

From the above findings, our preliminary investigation into demographic factors impacting equine vaccination suggest that it is the less active owners who do not travel, ride or compete with their horses that are least likely to vaccinate their horses against influenza and tetanus. In the case of influenza, older people with more horses, those in the breeding industry and donkey owners appear to be less likely vaccinate. Respondents under 16, those that don't

transport their horses, men and non-riders appear less likely to vaccinate against tetanus. Dramatically fewer respondents to our questionnaire vaccinated against herpesvirus compared to influenza or tetanus. Regional factors influenced vaccination coverage for all three diseases.

To conclude, a preliminary assessment of the results of this web-based questionnaire endorses it as a powerful tool for analysis of British equine demography. The overall vaccination coverage for influenza and tetanus of 90.3% and 95.1%, respectively, paint a positive picture of equine vaccination practice amongst British horse owners. However, our analyses have highlighted possible demographic variables where, after corroboration with multivariable analyses, should be focussed upon to improve vaccination coverage.

Chapter 3: Characterisation of G proteins in myelin isolated from the *rumpshaker* mouse model of Pelizaeus-Merzbacher disease

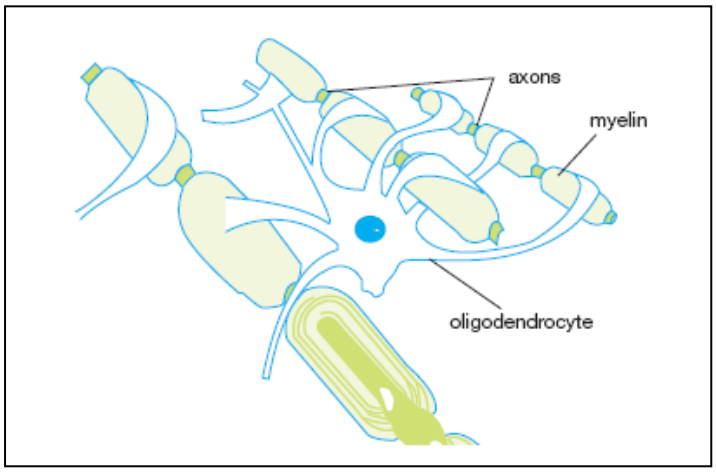
3.1 Background

3.1.1. The role of myelin in the central nervous system

3.1.1.1. Function

The nervous system is composed of specialised excitable cells called neurons that receive stimuli and transmit electrical impulses from one part of the body or nervous system to another. In addition, a range of supporting cells collectively referred to as neuroglia, and resident immune cells called microglia constitute up to 90% of the cells in the central nervous system. Each neuron transmits signals to other neurons or to its end target (e.g. a muscle fibre in the case of an efferent nerve or to other neurons in the sensory cortex in the case of an afferent nerve) *via* a synapse. Neurons have specialised extensions called axons to transmit stimuli from one area to another. The speed of conduction is significantly enhanced by specialised glial cells (oligodendrocytes in the case of the central nervous system and Schwann cells in the peripheral nervous system) that provide axonal insulation by spirally wrapping their membrane processes around axons during development to form a myelin membrane (Pedraza *et al*., 2001) (Figure 3.1). Myelination greatly increases the speed of impulses by promoting saltatory ("leaping") conduction of the stimulus between the unmyelinated segments (the nodes of Ranvier). As well as axonal insulation, myelin is essential for normal axonal transport of membranous organelles and proteins (Edgar *et al*., 2004b) with neurodegeneration occurring when myelin is absent or defective due to disease processes (Yin *et al*., 2006). However, not all axons are myelinated. Unmyelinated neurons tend to contain small diameter axons that are involved in specific functions such as pain reception (Julius and Basbaum, 2001).

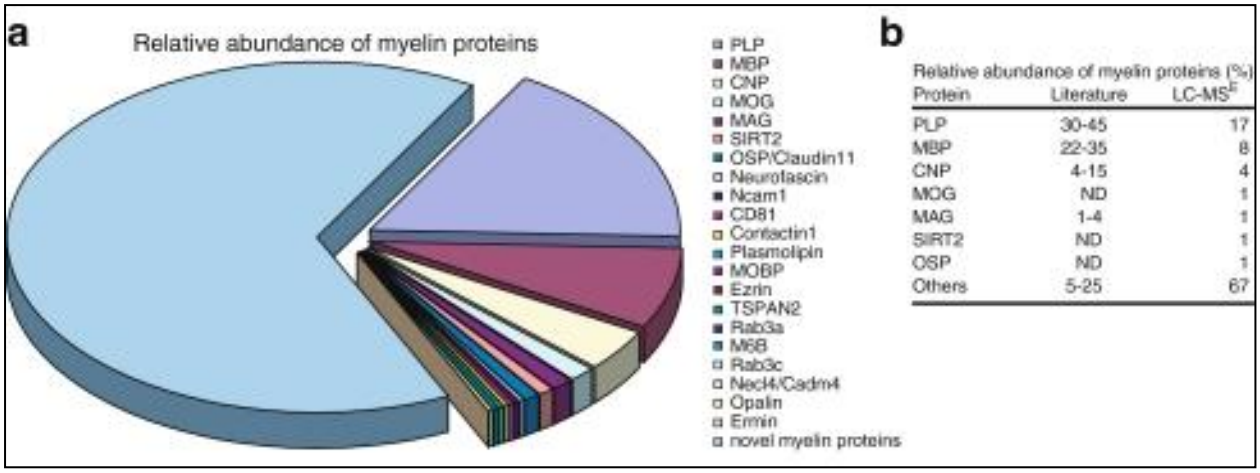
Figure 3.1: Oligodendrocyte and axons. Plasma membrane extensions from one oligodendrocyte can spirally wrap around multiple axons and a single axon may be myelinated by multiple oligodendrocytes.



3.1.1.2. Myelin components

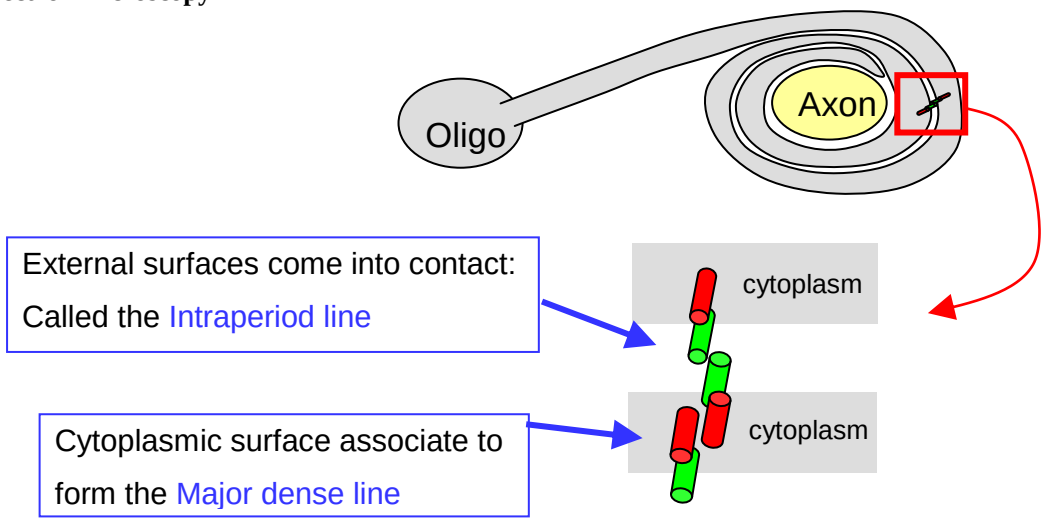
Myelin consists of 40% water. Of its dry weight, lipids are the most abundant component, making up 70%. The remaining 30% of dry weight is protein. The two most abundant myelin proteins are proteolipid proteins (PLP/DM20) and myelin basic protein (MBP), making up 17 and 8% of total myelin protein respectively (Jahn *et al*., 2009). The remaining protein content consists of several classical myelin proteins as well as a large selection of proteins newly identified in myelin using advances in proteomic techniques (Figure 3.2).

Figure 3.2: The relative abundance of myelin proteins, adapted from (Jahn *et al*., 2009)



Myelin is a highly organised laminar arrangement generated by the association of the extracellular and the intracellular cytoplasmic surfaces of the processes formed with each spiral wrap. This generates layers defined as the intraperiod and major dense line that can be identified by electron microscopy. The formation and maintenance of this highly specialised structure is dependent on the special and temporal orchestration of protein and lipid assembly into the myelin sheath (Quarles *et al*., 2006). (Figure 3.3)

Figure 3.3. Schematic illustration of the formation of the myelin sheath. The spiral wraps generates the intraperiod line and the major dense line sheath that can be visualised by electron microscopy



3.1.2. Pelizeus Merzbacher Disease

The clinical symptoms of Pelizeus Merzbacher disease (PMD) were first described as early ataxia, nystagmus (abnormal eye movement), uncoordinated limb movement and developmental delay in the males of one family (Pelizaeus, 1885). Whilst mostly males were affected, it was the females from the family who carried the disease from one generation to the next indicating an X-linked genetic basis. A histopathological examination of post-mortem nervous tissue from an affected member of the same family revealed diffuse hypomyelination in the central nervous system (Merzbacher, 1910). These early findings are consistent with what is now known about PMD; it is a genetic (recessive X linked) disorder associated with dysmyelenation (abnormal formation of myelin) in the central nervous system. The most frequent genetic abnormality is duplication of the Xq22 chromosome including the region encoding proteolipid protein 1 (PLP). However, deletions, triplications and mutations of the *PLP-1* gene also cause PMD (Woodward, 2008).

PLP and its slightly smaller isoform DM20 make up 17% of all myelin protein (Jahn *et al*., 2009). Whilst *Plp* mutations can result in a dysmyelinating phenotype and significant mortality, in the study of Klugman *et al*. (1997) PLP null mice were not demyelinated. The authors of that study did notice that the intraperiod lines in the myelin remained condensed, suggesting reduced physical stability of the myelin and a "zipper" like function for PLP. The study of Rosenbluth *et al*. (1996) also concluded that PLP is not essential for myelination but is necessary for normal stability of the myelin sheath after formation. This finding corroborates that of (McLaughlin *et al*., 2006) on PLP processing in *rumpshaker* mice suggesting that it is an alteration in or an excess of PLP rather than a deficiency of PLP which somehow results in the dysmyelinating phenotype. Despite PLP null mice being able to form myelin sheaths, Edgar *et al*. (2004) have shown compromised axonal transport of organelles and proteins in axons with overlying PLP null myelin and Yin *et al*. (2006) reported that PLP in myelin is essential for maintenance of axonal integrity. Hence, many cellular functions of PLP have yet to be definitively elucidated. Hypomyelination in PMD causes a spectrum of symptoms, from mild ataxia, tremor and spasticity of the lower limbs (hereditary spastic paraplegia) to complete loss of muscle tone, seizures and premature death (severe connatal PMD).

3.1.3. The rumpshaker mouse model of Pelizeus Merzbacher Disease

The spontaneous *rumpshaker* ($Ile^{186}Thr$) mis-sense mutation of the *Plp-1* gene in both man and C3H mouse generates misfolded PLP protein resulting in dysmyelination. Phenotypically, there are mild symptoms of PMD or spastic-paraplegia type 2 (Griffiths *et al*., 1998; Klugmann *et al*., 1997) Woodward, 2008). The C3H laboratory mouse type with the *rumpshaker* mutation exhibits ataxia and tremor but has normal longevity and the tremor diminishes as the mice age beyond 4 months. However, in aged mice beyond 1 year of age an axonopathy develops supporting the role of PLP in maintaining axonal integrity (Edgar *et al*., 2004a). It was also observed that changing the genetic background of the *rumpshaker* had a profound effect on the phonotype. When this mutation occurs in a C57/BL6 background, hypomylination is more severe and mice are prone to seizures and premature death (Al-Saktawi *et al*., 2003).

3.1.4. PLP dynamics in the rumpshaker mouse model

The PLP content of *rumpshaker* myelin is markedly reduced compared to wild type and other myelin proteins included MBP are also reduced. It has been shown that the low steady state levels of PLP in the *rumpshaker* model are primarily the result of increased degradation rather than reduced synthesis (McLaughlin *et al*., 2006). There is a cellular unfolded protein stress response and sub-normal amounts of PLP are integrated into the cell oligodendrocyte membranes (Griffiths *et al*., 1998; McLaughlin *et al*., 2006; McLaughlin *et al*., 2007;Barrie *et al*., 2010). McLaughlin *et al*. (2007) reported that there was no difference in the PLP dynamics in C3H compared to C57 *rumpshaker*s, indicating that this did not play a role in the dramatic difference in the phenotypes of the *rumpshaker* mutation on C3H and C57 genetic backgrounds. The restoration of the PLP/DM20 content of *rumpshaker* myelin with wild-type *Plp* through genetic complementation (*rumpshaker* heterozygote females were crossed with male mice carrying a transgene that contained increased doses of the *Plp-1* gene) improved the phenotype and myelin integrity. However, hypomyelination persisted and stress pathways remained activated, suggesting that the *rumpshaker* was associated with a toxic gain of PLP function as well as a loss of function (Barrie *et al*., 2010). Interestingly, the levels of myelin basic protein (MBP), which is essential for normal myelination and myelin compaction, are low in *rumpshaker* mice. MBP levels were not restored at peak myelination (P20) but did recover at P60 with transgenic complementation with wild-type *Plp* in the *rumpshaker* models in the study of (Barrie *et al* 2010). This suggests that it is the toxic gain of function of *rumpshaker* PLP influences the co-ordinated assembly of MBP into the myelin sheath although a loss of function may also affect MBP levels. It is possible that increased turnover of misfolded PLP (as shown by Mc Laughlin *et al* 2006) is overloading the organelles responsible for protein degradation and synthesis (as evidenced by the prominent endoplasmic reticula and golgi complexes described by (Griffiths *et al*., 1990). This may compromise the synthesis and transportation of other proteins such as MBP.

3.1.5 Myelin Basic Protein

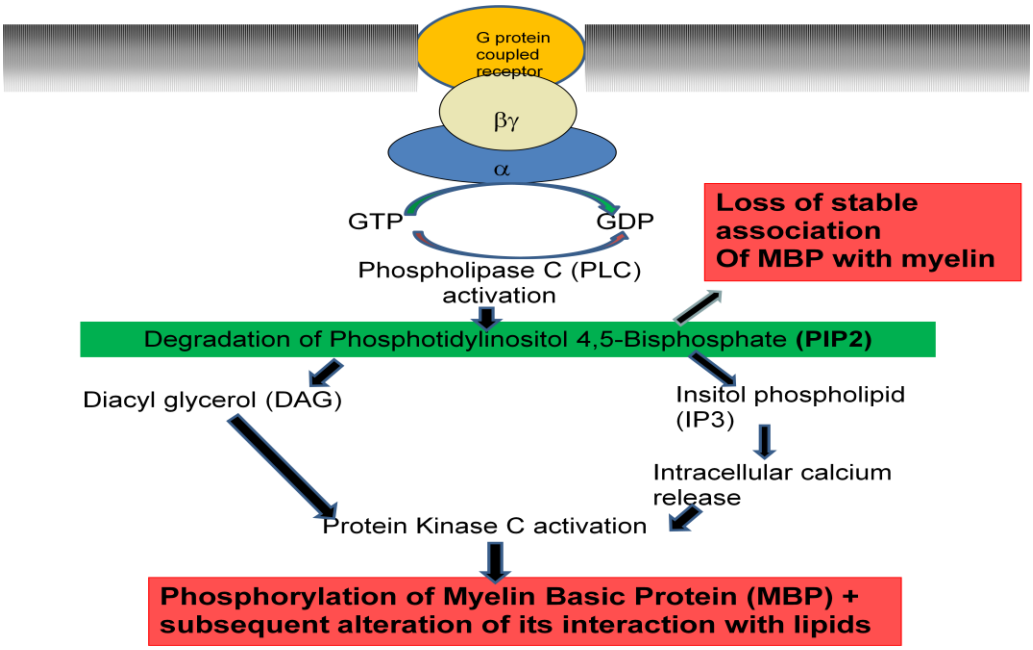
MBP is the second most abundant myelin protein after PLP, making up 8% of all myelin protein (Jahn *et al.*, 2009) and is required for normal myelination (Readhead *et al*., 1987). The myelin-deficient mutant *shiverer* mouse lacks basic protein (MBP) in the myelin of its central nervous system and causes sever hypomyelination MBP interaction with the cytoplasmic leaflets of the myelin processes is thought to cause two opposing leaflets to

physically associate, resulting in myelin membrane compaction (Omlin *et al*., 1982) and Readhead *et al* 1987. This is facilitated by the high positive charge of MBP and negative charge of cytoplasmic membranes (Harauz *et al*., 2004) Furthermore, MBP is believed to be involved with lipid packing and organisation in the membrane which is important for membrane assembly and signalling events (Fitzner *et al*., 2006)(MBP is significantly reduced in the *rumpshaker* mouse (Al-Saktawi *et al*., 2003).

3.1.6. Phosphotidylinositol 4,5-Bisphosphate (PIP2)

PIP2 is the major polyphosphoinositide in mammalian cells. It has a myriad of functions including being a lipid anchor in the plasma membrane and attachment of the cytoskeleton to the plasma membrane, interaction with many different proteins, endo and exocytosis and membrane trafficking of proteins. It is believed that the non-uniform distribution of PI2 in the lipid membrane is what enables this spectrum of functions. An important role of PIP2 is as the source of two second messengers in the cell; diacylglycerol (DAG) and inositol1,4,5-trisphosphate (IP3) (McLaughlin *et al*, 2002). Cellular phospholipase C (PLC) and phosphoinositide 3-kinase (PI3-kinase) breaks PIP2 down to produce DAG and IP3(Berridge, 1993). These second messengers stimulate a variety of protein kinases and intracellular calcium release. Activation of the $G_{\alpha q}$ subunits of heterotrimeric G proteins by G protein coupled receptor agonist binding on the plasma membrane activates PLC mediated breakdown of PIP2. A pilot study by Dr. Mark Mclaughlin has shown significantly decreased levels of PIP2 in C3H *rumpshaker* mice (Appendix Figure A.4).

Figure 3.4. Schematic illustration of a potential signalling cascade involving PIP2 that influences MBP association with the myelin sheath



3.1.7. Interactions between PIP2 and MBP

A crucial interaction between PIP2 and MBP has recently been reported (Nawaz *et al*., 2009). When PIP2 was hydrolysed, it caused dissociation of MBP from the plasma membrane. Furthermore, when PIP2 enriched endosomal vacuoles were induced in a primary oligodendrocyte culture, MBP redistributed to these vacuoles. PIP2 has a negative charge which lends itself to MBP docking (McLaughlin *et al*., 2002). The IP3 product of PIP2 breakdown causes increased intracellular calcium release which also results in dissociation of MBP from the glial cell plasma membrane (Nawaz *et al*., 2009). The PLC PIP2 hydrolysis pathway also results in the activation of protein kinase C which catalyses the phosphorylation of MBP (Kishimoto *et al*., 1985). Phosphorylated MBP is likely to have an altered interaction with lipids present in the myelin sheath.

3.1.8. G proteins

Guanine nucleotide binding (G) proteins propagate incoming messages from receptors to effector proteins, switching from an inactive to active state by exchanging a GDP molecule for a GTP molecule. In a dormant state, G-proteins are composed of three subunits, α, β and γ. Upon activation, heterotrimeric G proteins dissociate into α and βγ subunits which migrate to membrane microdomains to activate their target proteins (Vögler *et al*., 2008). There are 16 G protein α-subunits, five β and 14 γ encoded on the mammalian genome (Milligan and Kostenis, 2006). G protein sub-types are involved in the signalling pathways for a diverse spectrum functions. The $G_{\alpha q}$ subunit activates PLC mediated breakdown of PIP2 and the DAG and IP3pathway (Gilman, 1987). Another α subunit, $G_{\alpha o}$ has been found in high quantities (1% of membrane protein) in bovine brains (Sternweis and Robishaw, 1984), with $G_{\alpha o}$ deficient transgenic mice showing multiple neurological deficits (Jiang *et al*., 1998). $G_{\alpha o}$ is known to regulate potassium and calcium ion release as well as activating adenylate cyclase and is integral to cellular responses to neurotransmitter-receptor binding (Jiang and Bajpayee, 2009)

$G_{\beta\gamma}$ subunits have a plethora of targets and actions also, including potassium and calcium channel regulation, phospholipase C activation, adenyl cyclase and MAP kinase activation (Clapham and Neer, 1997) as well as more recently identified interactions with receptors within the Golgi apparatus and endoplasmic reticulum and modulation of the activity of G_{α} subunit activity (Dupre *et al*., 2009)

The possible influence of $G_{\alpha q}$ and $G_{\beta\gamma}$ proteins on PIP2 levels (and subsequently MBP function) through the PLC pathway is clear (Figure 3.4). However, it is only a recent pilot study that highlighted the altered PIP2 levels in *rumpshaker* mice (Mark McLaughlin, unpublished observations in appendix.) The foremost question in the wake of these findings with PIP2 is whether G protein levels altered in the myelin of *rumpshaker* mice. G proteins and their G protein coupled receptors have been detected in myelin (Jahn *et al*., 2009). However, to our knowledge, no previous study has measured G protein levels in the myelin of *rumpshaker* mice.

The results of our study show significantly increased levels of $G_{\alpha o}$ and $G_{\alpha q}$ in the myelin of C3H *rumpshaker* mice compared to that of matched wild-type controls. Ancillary

investigations comparing the levels of MBP, MAG, HSP90 and ASPA indicate that this result is unlikely to be technical issue associated with the isolation of myelin from a hypomyelinated mouse.

3.2. Aims and Objectives

This project aims to ascertain if $G_{\alpha q}$, which is involved in the PIP2 degradation and intracellular calcium release, is more abundant in mice with the *rumpshaker* mutation. An ancillary aim is to characterise the levels of $G_{\alpha o}$, and abundant protein in central nervous tissue in the myelin extracted from *rumpshaker* and wild-type mice.

Specifically, our objectives are to:

1. Ascertain if $G_{\alpha o}$, $G_{\alpha q}$ and G_{β} can be detected in the myelin and other central nervous system fractions of *rumpshaker* and wild-type mice.
2. Measure and compare $G_{\alpha o}$, $G_{\alpha q}$ and $G_{\beta\gamma}$ in *rumpshaker* and wild-type mice.
3. Determine if there is any association between the G-protein levels and the extend of hypomyelination using mouse models with a spectrum of hypomyelination

3.3. Materials and methods

3.3.1 Breeding and genotyping of mice *(Performed by Dr Mark McLaughlin and Jennifer Ann Barrie).*

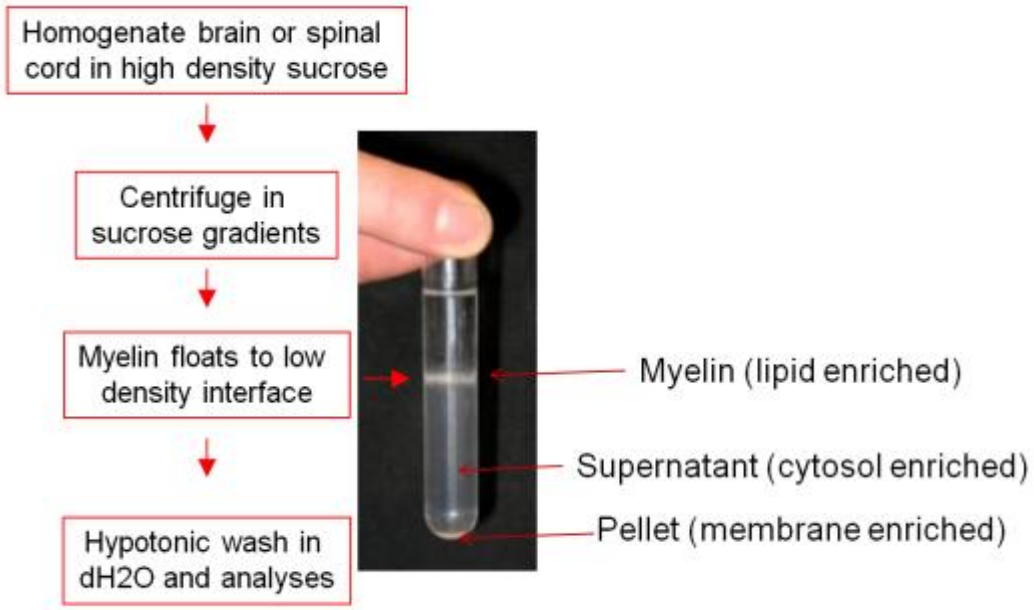
The *rumpshaker Plp1* (Ile^{186}Thr) mis-sense mutation occurs spontaneously on the genetic background of C3H/HeH crossed with 101H mice and has been maintained on a C3H/101 hybrid background (Griffiths *et al*., 1990). It is now referred to as C3H . In order to impose this mutation on C57/BL6 genetic background (now referred to as C57), female *rumpshaker* heterozygotes on a C3H background were crossed with C57 males. The female heterozygous offspring were then back-crossed with wild-type C57 males 6-10 times, resulting in a *rumpshaker* mutation on a C57 background. The backcrossing of *rumpshaker* onto a C57 background was conducted to unify the genetic background of the *rumpshaker* with the transgenic mouse line #66 that harbours additional copy numbers of the wild type *Plp1* gene. This would permit a genetic complementation study to restore the level of wild type PLP in the *rumpshaker* by crossing heterozygote female *rumpshaker* with male mice containing the *Plp1* trangene. *Rumpshaker* mice were identified by their clinical signs and PCR genotyping and restriction digest, as the *rumpshaker* mutation contains a site for the AccI restriction enzyme (Schneider *et al*., 1992). Only affected male mice and their wild type littermates were used for this present study. Transgenically complemented mice were identified by PCR using primers based on the T7 viral promoter that is associated with the PLP transgene and the 3' flanking region of the *Plp* gene (Readhead *et al*., 1994). The mice were killed by CO_2 overdose, their brains and spinal cords were rapidly removed and snap frozen in liquid nitrogen until required. Spinal cord samples only were used for this particular study due to the greater degree of homogeneity in their cellular and myelin content. All animal studies were approved by the Ethical Committee of the University of Glasgow and licensed by the U.K. Home Office.

3.3.2 Myelin extraction *(Performed by Jennifer A Barrie)*

Myelin was extracted from spinal cords from one wild type mouse or two pooled mutant mice per experiment. The spinal cord tissue was added to 0.85 Molar sucrose (Sigma-Aldrich) containing 1mM concentration of both sodium pyruvate and sodium orthovanadate (phosphatase inhibitors) and a protease inhibitor cocktail (Sigma-Aldrich). Following

homogenization and high speed centrifugation, myelin was collected from the interface of 0.25/0.85M sucrose and subjected to 3 rounds of osmotic lysis as described by Al-Saktawi *et al*. (2003) (Figure 3.5). The solutions were kept on ice throughout the myelin extraction. The end products of the myelin preparation were myelin, supernatant (cytosol enriched) and pellet (membrane enriched) fractions. Samples of the total spinal cord homogenate were also taken. All samples were stored at -20^{o}C until further processing.

Figure 3.5: Schematic outlining the myelin extraction protocol



3.3.3. Protein assay

The concentration of protein in each sample was calculated using a commercial assay kit (Pierce® BCA Protein Assay Kit) which is based on bicinochoninic acid (BCA) and the biuret reaction for the colorimetric detection and quantification of total protein. The protein assay was conducted according to the manufacturer's instructions.

3.3.4. Sodium dodecyl(lauryl)sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The separation of proteins was performed using SDS-PAGE electrophoresis. These analyses were performed using precast Nu-Page gels purchased from Invitrogen. The samples were prepared to a total volume of 21µl including 7µl of 3x concentration sodiumdodecylsulphate/dithiothreitol (SDS/DTT) denaturing buffer and heated to 90ºC for 4 minutes, to linearise the protein and allow SDS association at a uniform charge to protein

ratio. Ten or twelve one mm well Invitrogen 4-12% gradient gels were loaded with samples together with the SeeBlue® Plus2 pre-stained molecular weight standards (Invitrogen) as references for the molecular weight of the proteins. Electrophoresis was conducted in a MES SDS buffer at a constant voltage (135 Volts) for one hour. After PAGE, the gels were either stained with silver stain or electro-transferred to nitrocellulose membranes for western blotting.

3.3.5. Silver staining

In order to gain a visual appreciation of the protein profile of each sample, the proteins were stained using a sensitive silver-staining protocol. This method permitted a comparison of the profile between the various genotypes and also provide an index of the consistency of the amount of protein loaded onto the gel. The Invitrogen Silverexpress® system was used according to manufacturer's instructions. Initially, for the earlier C3H samples, 2μg of each sample was loaded onto the PAGE gel for electrophoresis prior to silver staining. However, due to heavy staining of the samples, this was reduced to 1 μg of each sample for the latter C57 samples.

3.3.6. Samples used

As a pilot study, spinal cord fraction samples from a single representative each genotype were subjected to western blotting using G-protein antibodies. When it was established that G-proteins antibodies can react with the specific proteins in myelin, the case-control study commenced. Pellet fractions from 4 C3H wild-types, 4 C3H *rumpshaker*s, 4 C57 wild-types, 4 C57 *rumpshaker*s and 4 C57 *rumpshaker* mice containing the Plp1 trangene were subjected to SDS-PAGE and silver staining. Myelin fractions from the same mice were loaded onto a separate gel and subjected to the same procedure. The C3H and C57 samples were loaded to separate gels.

3.3.7. Western blotting

The above samples were loaded onto polyacrylamide gels and subjected to electrophoresis. Electrotransfer of proteins onto nitrocellulose membranes was performed using the Invitrogen iBlot® Gel Transfer system. After checking for successful transfer of proteins with Ponceau-S staining of the nitrocellulasoe membranes, non-specific binding sites were blocked with 5% skimmed milk, for 1 hour at room temperature. Membranes (blots) were then incubated with

antibodies against $G_{\alpha q}$, $G_{\alpha o}$, $G_{\beta\gamma}$ MBP and myelin associated glycoprotein (MAG) suspended in 5% skimmed milk at 4 degrees overnight on an orbital shaker. (See Tables 3.1 and 3.2 for antibody details.) The blots were then irrigated three times with tris buffered saline containing 0.05% tween 20 (T-TBS) and incubated with the appropriate horse-radish-peroxidase (HRP) coupled secondary antibody (suspended in 5% skimmed milk) for 2 hours. The irrigation procedure was repeated to remove excess and secondary antibody and peroxidase substrate for enhanced chemiluminescence (ECL) was applied to the blots Thermo-Scientific). (The HRP on the bound secondary antibody catalyses the transformation of ECL to its sensitized form which then goes on to react with added peroxide to produce a product which emits light upon decay.) The blots were exposed to Amersham Hyperfilm ECL (GE healthcare) film for 30 second to 10 minute intervals. The intensity (signal) of the image made by the chemiluminescence emitted from the blots corresponded with the initial quantity of the protein of interest on the blot from each sample originally loaded in the gel. The chemiluminescent film was scanned and the pixel density of each image was quantified with Scion Image software (NIH).

T**able 3.1: Details of primary antibodies used in the study.**

Samples probed	1° Antibody	Isotype	Dilution	Source
C3H fractions, myelin, pellet C57 fractions, myelin pellet	$G_{\alpha q}$	Rabbit	1;1000	Gift from Prof. Graeme Milligan
	$G_{\alpha o}$	Rabbit	1;2000	
	$G_{\beta\gamma}$	Rabbit	1 in 750	
	MBP	Rabbit	1 in 25,000	Chemicon
	MAG	Rabbit	1;5000	Chemicon
C3H myelin and pellet	ASPA	Rabbit	1 in 2000	J Garbern (Gift)
	HSP 90	Mouse	1 in 10,000	Chemicon

Table 3.2: Details of secondary antibodies used in the study.

2° Antibody	Dilution	Source
Anti-rabbit HRP	1;10,000	Sigma
Anti-mouse HRP	1;5000	Sigma

3.3.8 Analysis of quantitative results

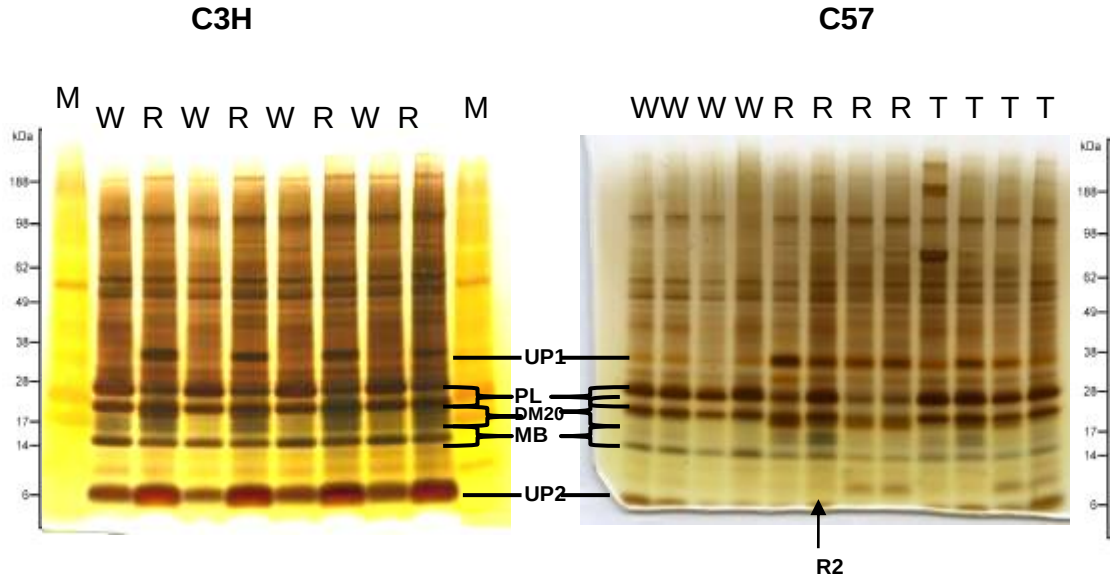
Statistical analysis of the protein data obtained from each group was compared by Student's T test was used where two sets of samples (C3H wild-type and *rumpshaker*) were being compared. Where three sets of samples (C57 wild-type, *rumpshaker* and *rumpshaker* transgene) were being compared, analysis of variance (ANOVA) and Bonferroni's multiple comparison test was used. Analysis was performed using Graphpad Prism 4 software (GraphPad software Inc, San Diego CA).

3.4 Results

3.4.1 The assessment of sample quality, the level of variation between samples and the profile of each genotype by silver staining

To assess the consistency of amount of protein loaded for each sample, protein profiles were visualised with silver staining. Intensity of protein staining and the pattern obtained for each group appeared consistent, reflecting acceptable accuracy of protein assays and gel loading. The identity of some classical myelin proteins including PLP/DM20 and MBP could be suggested by their relative abundance in myelin and their migration on SDS PAGE gels. Many unidentified proteins appeared to be similar between the wild-type and *rumpshaker* for the C3H mice and for the C57 mice. However, two protein bands, one of between 28 and 35 kDal (UP1) and one of under 15kDal (UP2) in size, appeared to have increased intensity of staining in the *rumpshaker* samples. One of the C57 *rumpshaker* samples (RSh2) appeared to have higher levels of PLP than expected. It is possible that this myelin came from a mis-genotyped C57 *rumpshaker* transgene mouse (Figure 3.6).

Figure 3.6: Polyacrylamide gels containing C3H and C57 myelin samples after electrophoresis and silverstaining. Proteolipid protein (PLP), DM20 and myelin basic protein (MBP) were identifiable as well as two unknown protein bands of between 28 and 35 kDals and less then 15 kDals (respectively) in size (UP1 and 2). C57 *rumpshaker* 2 (R2) had more intense silver staining representing PLP than would be expected for a *rumpshaker*. The silver staining in this sample is more consistent with the myelin of a *rumpshaker* transgene. W = wild-type, R = *rumpshaker*, T = *rumpshaker* transgene.



3.4.2 Assessment of the suitability of G-protein antibodies to conduct a Western blot analysis of myelin

It was important to determine if the G-protein antibodies could react with specific proteins in the samples and allow semi-quantitative comparison between genotypes. Pilot studies were therefore performed on a representative sample from each genotype to assess the intensity and specificity of the immunocomplex across all the fractions that are obtained during the myelin extraction procedure. 15ug of the total, supernatant and pellet fraction were analysed and 2µg of each myelin sample was analysed due to the limited availability of myelin from the *rumpshaker* genotypes.

3.4.2.1 $G_{\alpha o}$ in C3H spinal cord fractions

A robust chemiluminescent signal corresponding with a protein sized between 38 and 49 kDals was detected with 2µg samples of myelin and 15µg samples of the other fractions with $G_{\alpha o}$ anti-bodies. $G_{\alpha o}$ was enriched in the myelin of the *rumpshaker* but not of the wild-type (Figure 3.7).

3.4.2.2 $G_{\alpha q}$ in C3H spinal cord fractions

A robust chemiluminescent signal corresponding with a protein sized between 38 and 49 kDals was detected with 2µg samples of myelin and 15µg samples of the other fractions with $G_{\alpha q}$ anti-bodies. There was also non-specific binding to proteins in the total, supernatant and pellet fractions of the wild-type and the total and pellet fractions of the *rumpshaker* spinal cord which were over 49 kDals in size. $G_{\alpha q}$ was enriched in the myelin of the *rumpshaker* (Figure 3.8).

3.4.2.3 $G_{\beta\gamma}$ in C3H spinal cord fractions and in equine myelin

Western blot analysis of the various fractions with $G_{\beta\gamma}$ antibody, detected a faint non quantifiable chemiluminescent signal. Subsequently, a trial was performed with higher quantities of wild-type mouse and equine myelin that was available in large amounts. A quantifiable chemiluminescent signal was produced using 20µg of murine myelin (data not shown). Due to the limited of quantities of myelin available from the specifically bred, control-matched mice, further studies using $G_{\beta\gamma}$ antibody were abandoned for this project.

Figure 3.7: Western blot analysis to detect Gαo in C3H spinal cord fractions; total homogenate (T), supernatant (SN), pellet (P) and myelin (M) fractions of wild-type (W) and *rumpshaker* (R) mice. Proteins of between 38 and 49 kDal in size reacted with the $G_{\alpha o}$ antibody. For the myelin and other fractions, samples corresponding with 2µg and 15 µg of protein (respectively) were added to the polyacrylamide gel wells. The graph represents myelin signal values multiplied by 7.5 to make them comparable to the signals from the other fractions.

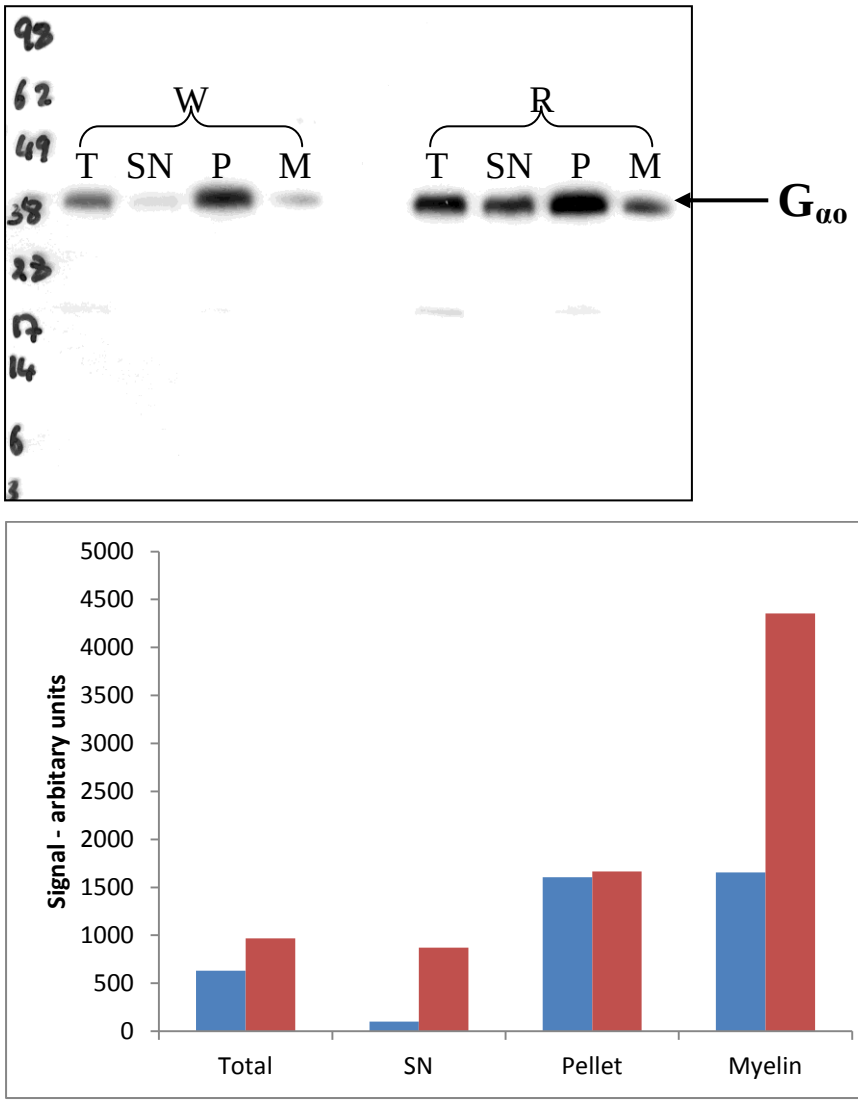
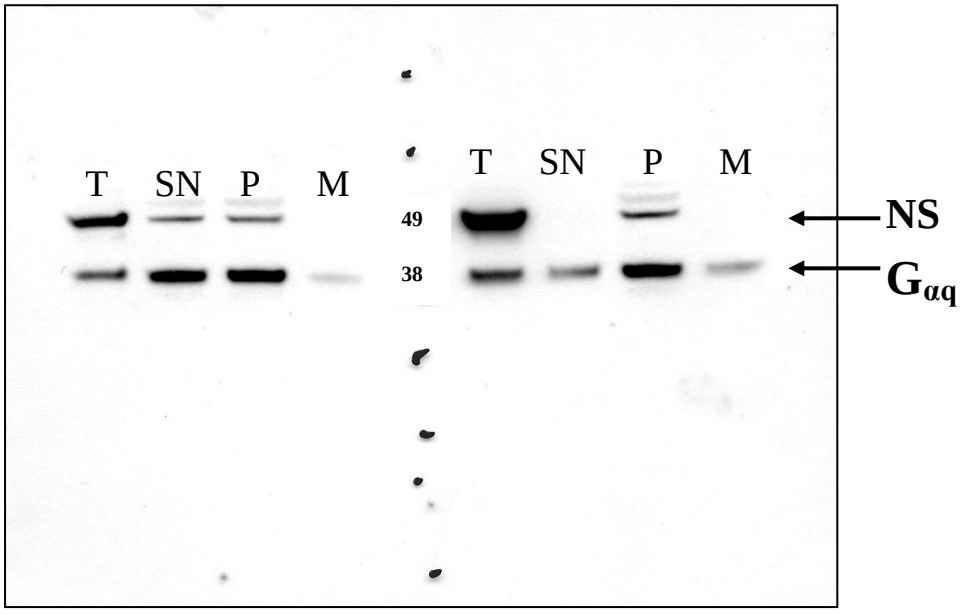
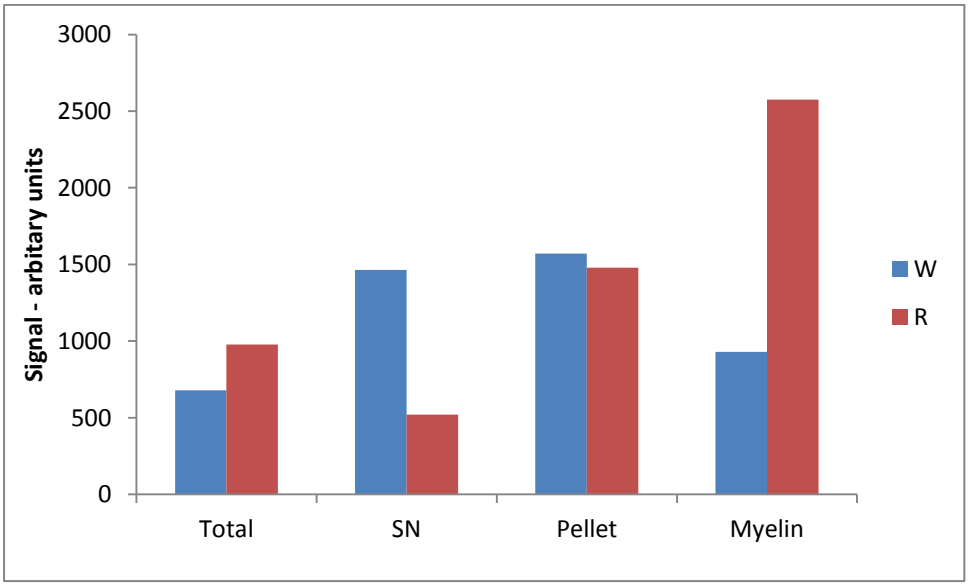


Figure 3.8: Western blot analysis to detect Gαq in C3H spinal cord fractions; total homogenate (T), supernatant (SN), pellet (P) and myelin (M) fractions of wild-type (W) and *rumpshaker* (R) mice. Proteins of between 38 and 49 kDal in size reacted with the $G_{\alpha q}$ antibody. There was also non-specific binding (NS) to proteins in the T, SN and pellet fractions of the WT and the T and P fractions of the RSh which were over 49 kDals in size. For the myelin and other fractions, samples corresponding with 2µg and 15 µg of protein (respectively) were added to the polyacrylamide gel wells. The graph represents myelin signal values multiplied by 7.5 to make them comparable to the signals from the other fractions.





3.4.3 Comparison of G-protein levels in the pellet fraction from C3H wild-type and rumpshaker mice.

The pellet fractions were analysed in the first instance since these fractions are rich in the plasma membrane component where the majority of G-proteins are located in all cells present in the spinal cord. This would provide some insight into the global expression of these proteins in the non-myelin fraction from the different genotypes

3.4.3.1 $G_{\alpha o}$ and $G_{\alpha q}$ in C3H pellet

$G_{\alpha o}$ and $G_{\alpha q}$ quantities were higher in C3H *rumpshaker* pellets but not significantly so (Figures 3.9 and 3.10).

Figure 3.9: Western blot analysis of Gαo in C3H wild-type (W) and *rumpshaker* (R) pellet indicated a non-significant increase in $G_{\alpha o}$ in the *rumpshaker* pellet samples.

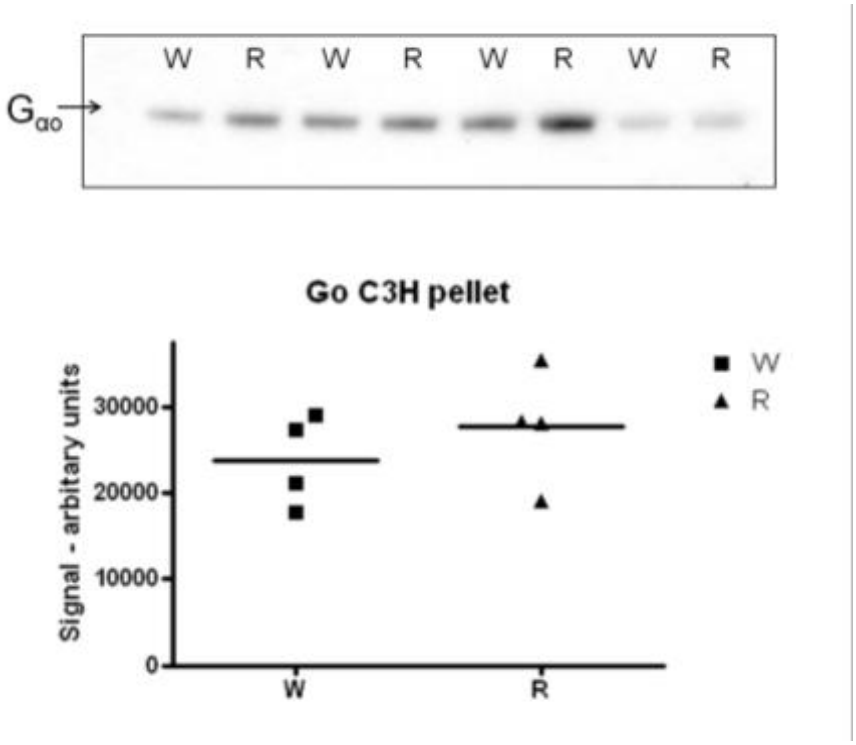
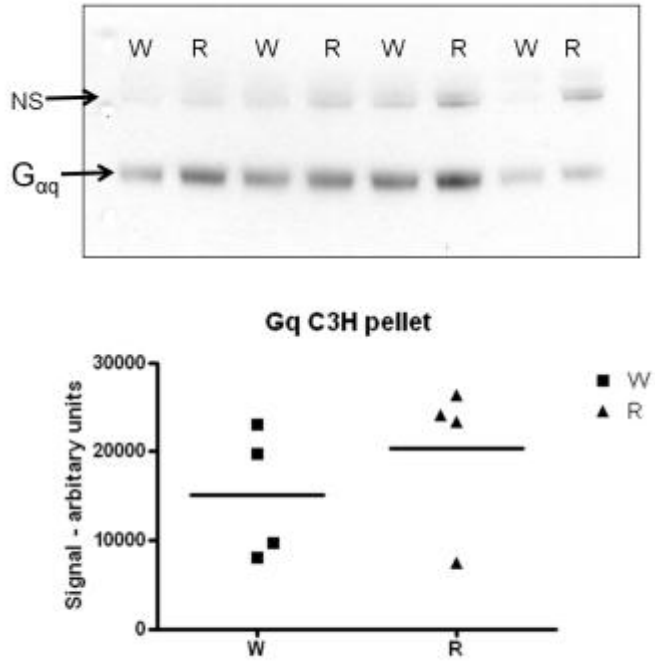


Figure 3.10: Western blot analysis of Gαq in C3H pellet fractions wild-type (W) and *rumpshaker* (R) mice indicated a non-significant increase in $nG_{\alpha q}$ in the *rumpshaker* pellet samples. Non-specific (NS) antibody binding was also evident.



3.4.4 Comparison of G-protein levels in the myelin fraction from C3H wild-type and rumpshaker mice

3.4.4.1 $G_{\alpha o}$ in C3H myelin

There was significantly more $G_{\alpha o}$ in C3H *rumpshaker* myelin compared to that of the wild-type mice (P-0.014) (Figure 3.11).

3.4.4.2 $G_{\alpha q}$ in C3H myelin

There was significantly more $G_{\alpha q}$ in C3H *rumpshaker* myelin compared to that of the wild-type mice (P-0.019) (Figure 3.12).

Figure 3.11: Western blot analysis of $G_{\alpha o}$ inC3H myelin fractions showing significantly increased $G_{\alpha o}$ levels C3H *rumpshaker* (R) myelin compared to wild-type (W) myelin. * denotes P<0.05 using Student's T-test where n=4).

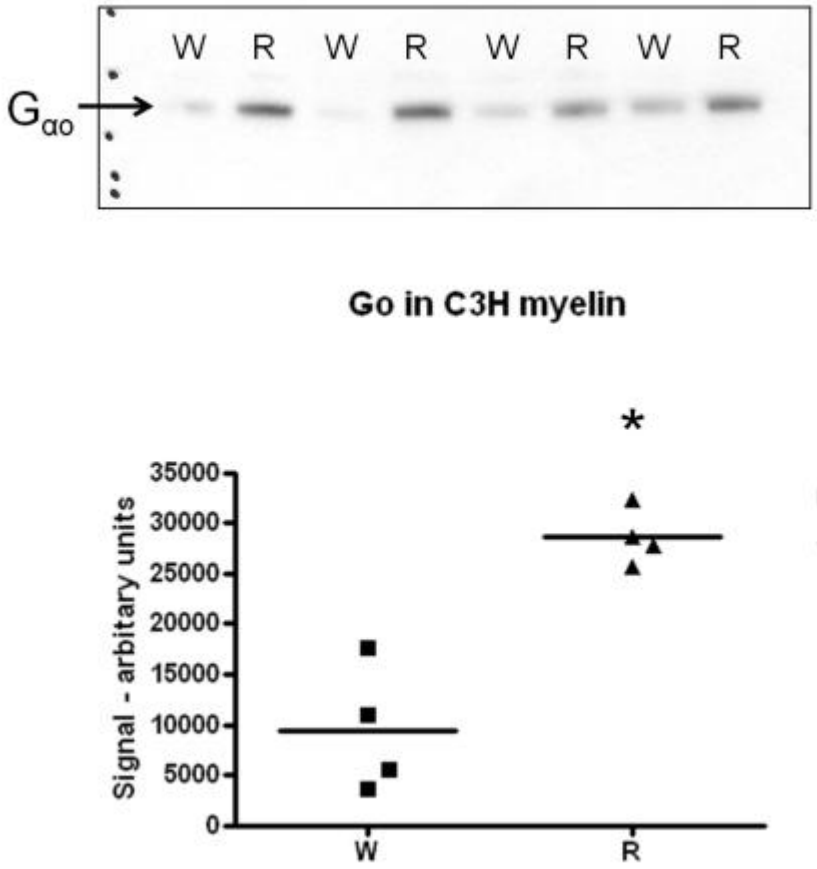
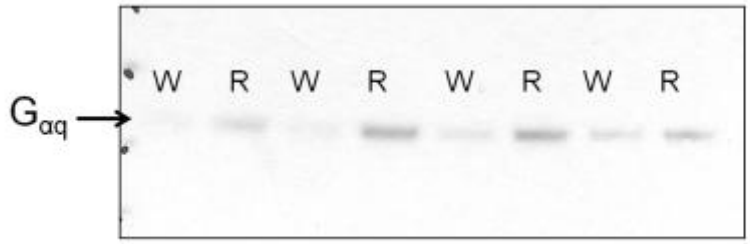
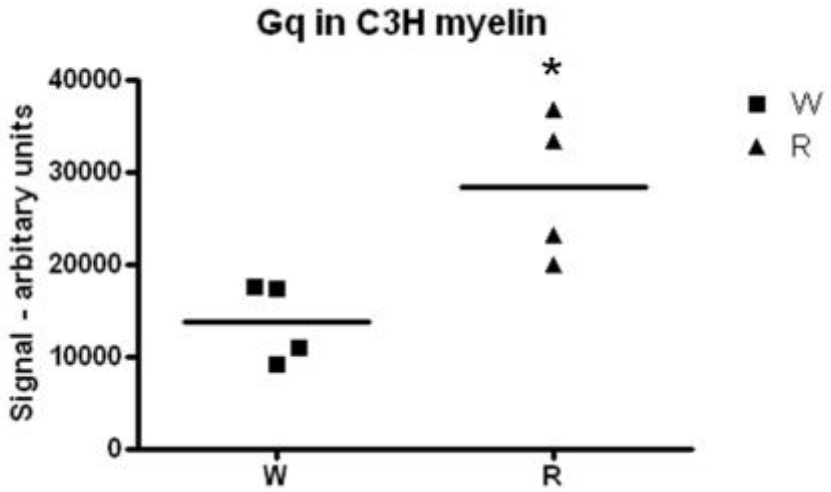


Figure 3.12: Western blot analysis of Gαq in C3H myelin fractions showing significantly increased $G_{\alpha q}$ levels in C3H *rumpshaker* (R) myelin compared to wild-type (W) myelin. * denotes P<0.05 using Student's T-test where n=4.





3.4.5 G proteins in C57 wild-type, rumpshaker and rumpshaker transgene

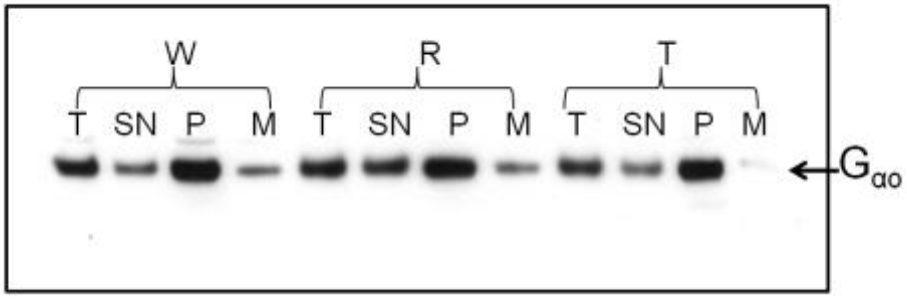
3.4.5.1 $G_{\alpha o}$ in C57spinal cord fractions

As with the C3H samples, subsequent to western blotting using anti-$G_{\alpha o}$ antibody, a robust chemiluminescent signal was detected corresponding with a protein sized between 38 and 49 kDals in 2µg samples of myelin and 15µg samples of the other fractions. $G_{\alpha o}$ was enriched in the myelin of the wild type and the *rumpshaker* but not of the *rumpshaker* transgene (Figure 3.13).

3.4.5.2 $G_{\alpha q}$ in C57 spinal cord fractions

A robust chemiluminescent signal corresponding with a protein sized between 38 and 49 kDals was detected with 2µg samples of myelin and 15µg samples of the other fractions with $G_{\alpha q}$ anti-bodies. There was also non-specific binding to proteins in the total, and pellet fractions of all three genotypes which were over 49 kDals in size. $G_{\alpha o}$ was enriched in the myelin of all three genotypes. (Figure 3.14).

Figure 3.13: Western blot analysis of Gαo in C57 spinal cord fractions of wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T) mice. For the myelin and other fractions, samples corresponding with 2µg and 15 µg of protein (respectively) were added to the polyacrylamide gel wells. The graph represents myelin signal values multiplied by 7.5 to make them comparable to the signals from the other fractions.



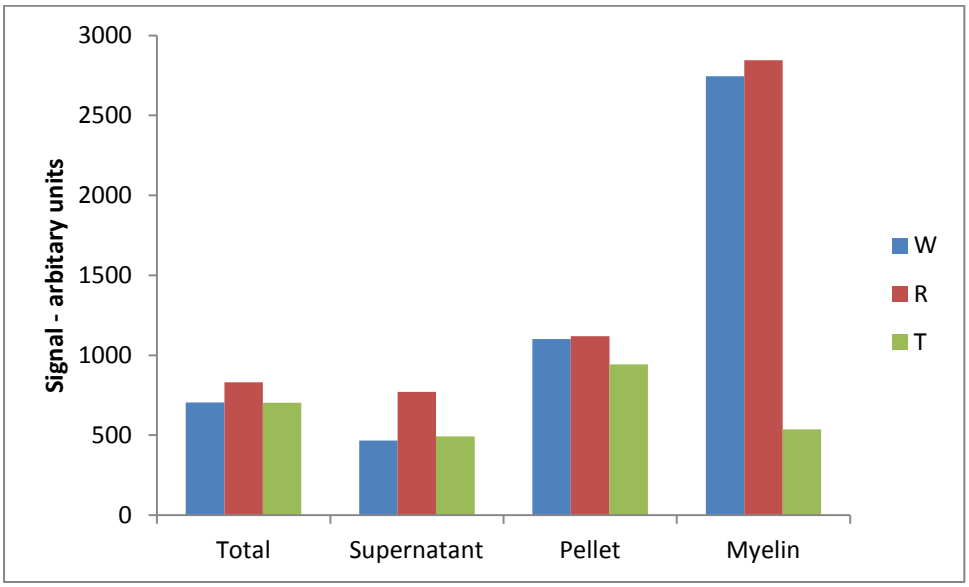
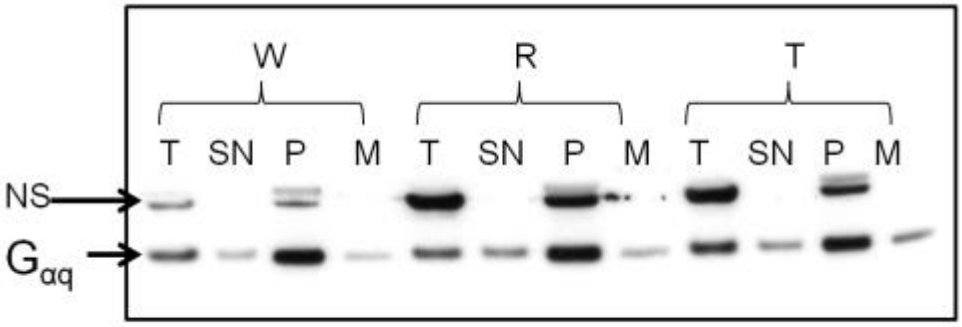
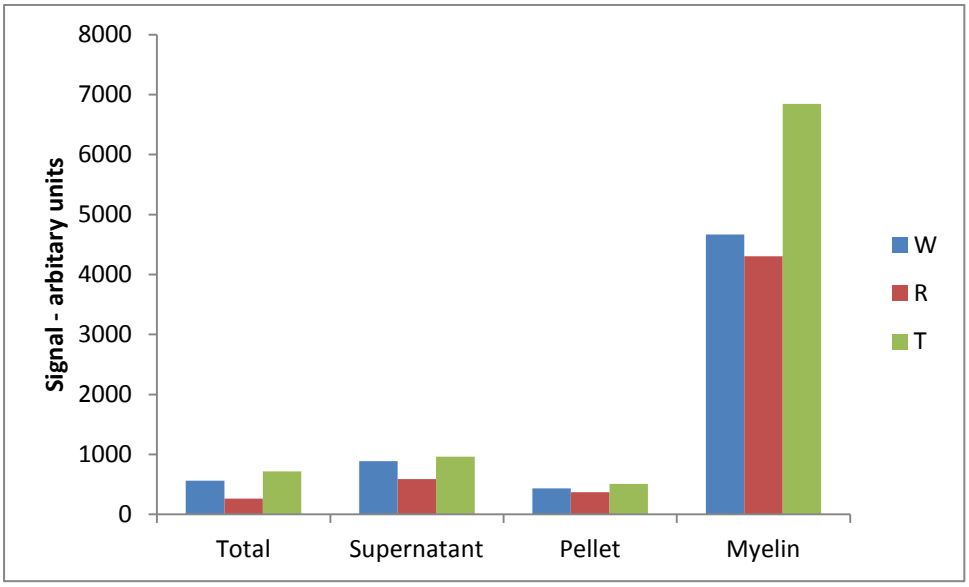


Figure 3.14: Western blot analysis to detect Gαq in C57 spinal cord fractions; total homogenate (T), supernatant (SN), pellet (P) and myelin (M) fractions of C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T) mice spinal cord preparations. Proteins of between 38 and 49 kDal in size reacted with the $G_{\alpha q}$ antibody. There was also non-specific binding (NS) to proteins in the T, SN and pellet fractions of the WT and the T and P fractions of the RSh which were over 49 kDals in size. The graph represents myelin signal values multiplied by 7.5 to make them comparable to the signals from the other fractions.





3.4.6 Comparison of G-protein levels in the pellet fraction from C57 wild-type, rumpshaker and rumpshaker transgene mice

3.4.6.1 $G_{\alpha o}$ in C57 pellet

There were no significant differences in $G_{\alpha o}$ levels in pellet samples from C57 wild-type, *rumpshaker* and *rumpshaker* transgene mice. Levels were reduced in C57 *rumpshaker* transgene pellet samples (but not significantly so) compared to wild-type and *rumpshaker* pellet samples (Figure 3.15).

3.4.6.2 $G_{\alpha q}$ in C57 pellet

There were no significant differences in $G_{\alpha q}$ in pellet samples from C57 wild-type, *rumpshaker* and *rumpshaker* transgene mice. $G_{\alpha q}$ levels were reduced in C57 *rumpshaker* transgene pellet samples (but not significantly so) compared to wild-type and *rumpshaker* pellet samples (Figure 3.16).

Figure 3.15: Western blot analysis of Gαo in C57 pellet fractions. There wasno significant difference in $G_{\alpha o}$ levels in pellet samples from C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T) mice. However there was a trend towards reduced $G_{\alpha o}$ levels in the *rumpshaker* transgene samples compared to the wild- type

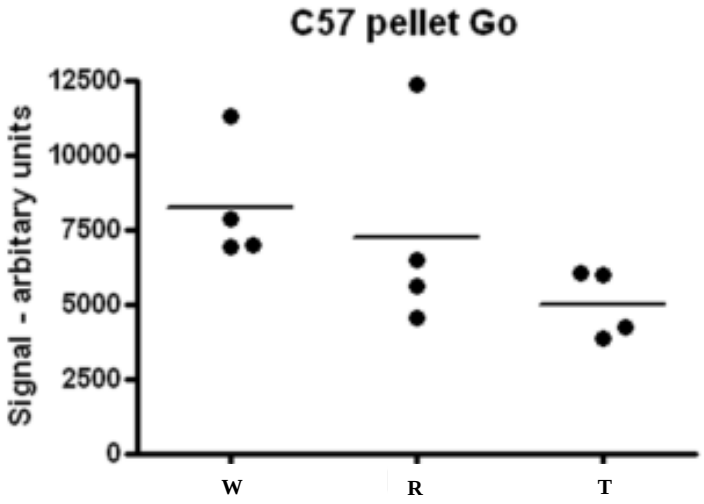


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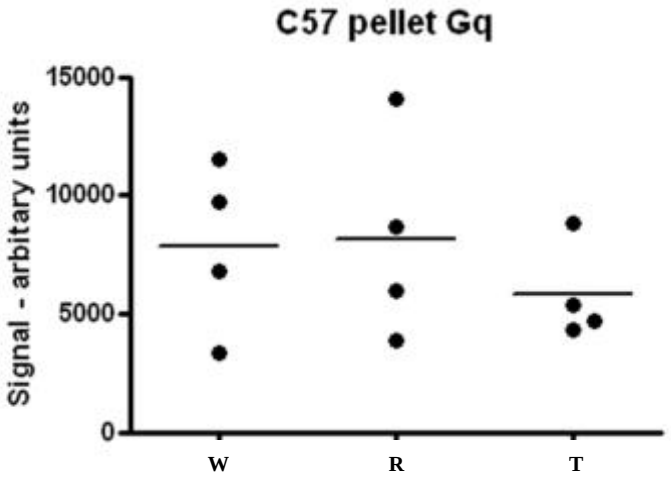
Figure 3.16: Western blot analysis of Gαq in C57 pellet fractions. There was no significant difference in $G_{\alpha q}$ levels in pellet samples from C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T) mice.



3.4.7 Comparison of G-protein levels in the myelin fraction from C57 wild-type, rumpshaker and rumpshaker transgene mice

3.4.7.1 $G_{\alpha o}$ in C57 myelin

There were no significant differences in $G_{\alpha o}$ in myelin samples from C57 wild-type, *rumpshaker* and *rumpshaker* transgene mice. However there was a trend towards increased $G_{\alpha o}$ the *rumpshaker* and *rumpshaker* transgene myelin compared to the myelin of the wild-type mice (Figure 3.17).

3.4.7.2 $G_{\alpha q}$ in C57 myelin

There were no significant differences in $G_{\alpha o}$ and $G_{\alpha q}$ levels in myelin or pellet samples from C57 wild-type, *rumpshaker* and *rumpshaker* transgene mice. However there was a trend towards increased $G_{\alpha q}$ levels in the *rumpshaker* and *rumpshaker* transgene myelin compared to wild-type myelin. CV as above (Figure 3.18).

Figure 3.17: Western blot analysis of Gαo in C57 ,myelin fractions. There was no significant difference between $G_{\alpha o}$ levels in myelin samples from C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T) mice. However there was a trend towards increased $G_{\alpha o}$ levels in the *rumpshaker* and *rumpshaker* transgene samples compared to the wild-type.

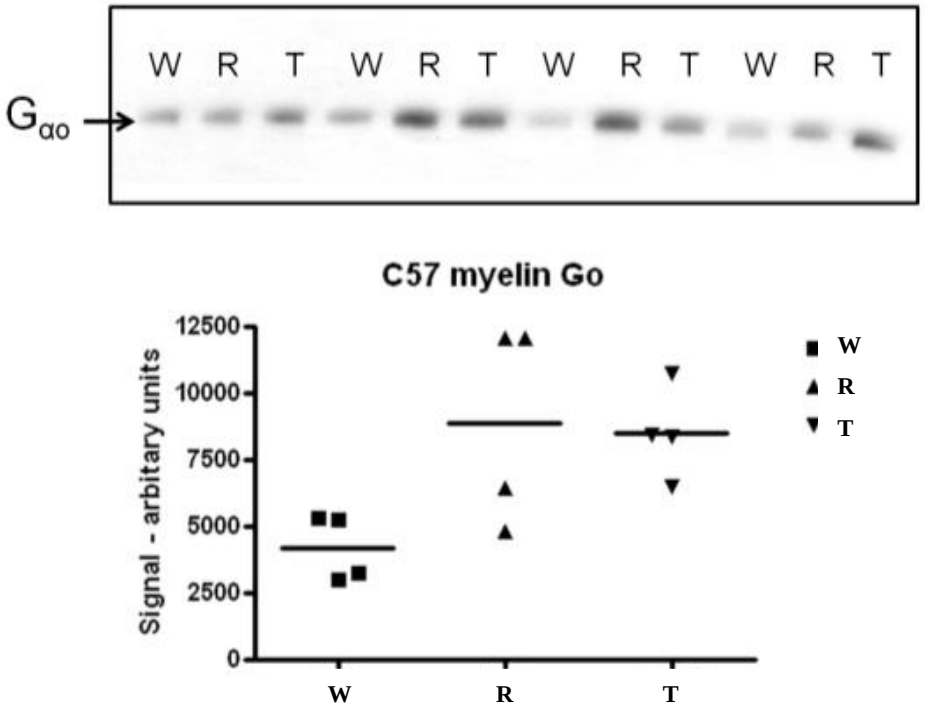
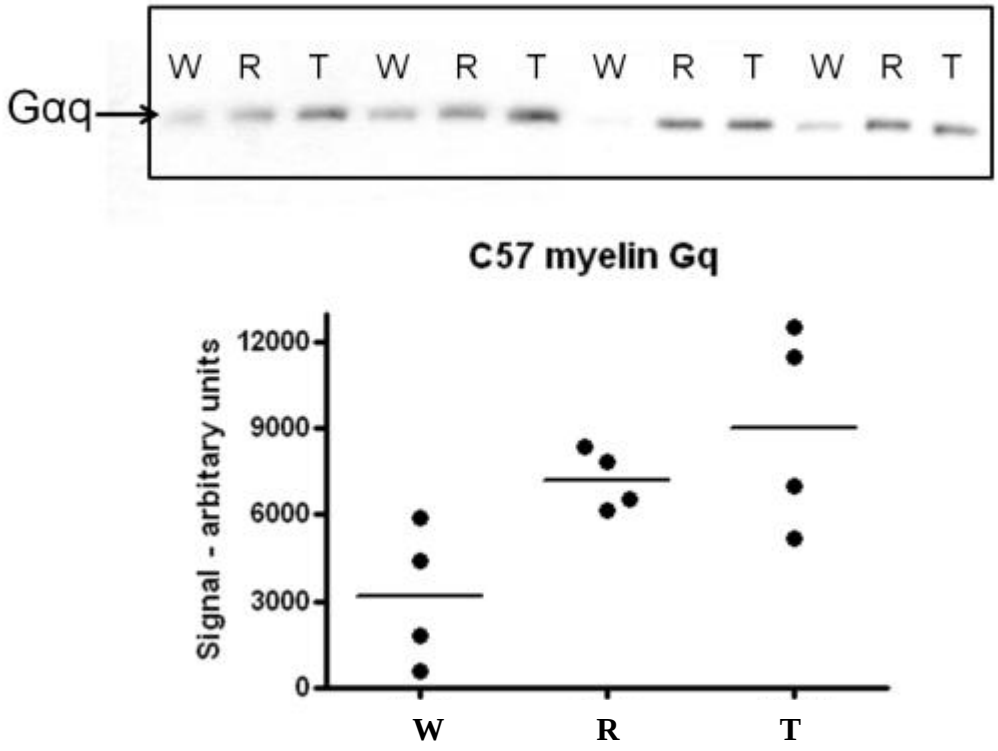


Figure 3.18: Western blot analysis of Gαq in C57 myelin fractions. There was no significant difference between $G_{\alpha q}$ levels in myelin samples from C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T, RShT) myelin. However there was a trend towards increased $G_{\alpha o}$ levels in the *RSh and RSh*T samples compared to the wild-type.



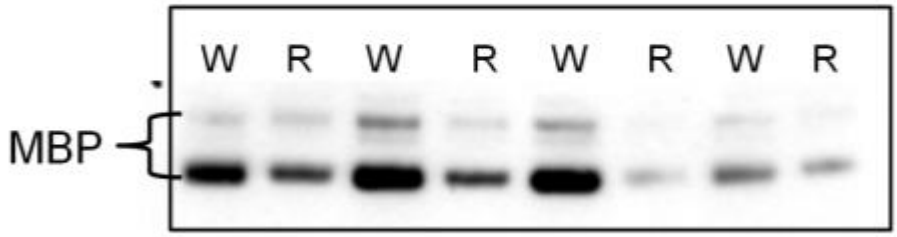
3.4.8 Exploring the possible impact of reduced myelin PLP in rumpshaker myelin

In addition to assessing the G proteins, western blots containing the samples were probed for MBP and MAG to control for any artificial relative increase in G proteins due to reduced PLP in *rumpshaker* mice. I.e. if there is less PLP (which makes up 17% of all myelin protein in a wild-type animal) will there be relatively more of other myelin proteins? If this was the case, MBP and MAG would be increased in the *rumpshaker* samples.

3.4.8.1 MBP in C3H and C57 myelin and pellet samples

MBP levels were significantly reduced in the pellet fractions of the C3H *rumpshaker* ($P=0.048$) compared to the wild-type. Also, in the C57 pellet samples, MBP was significantly lower in the *rumpshaker* and *rumpshaker* transgene compared to the pellet of the C57 wild-type ($P<0.001$). (Figures 3.19-3.22). However, analysis of the myelin fraction found that there was no significant difference in MBP levels between C3H wild-type and *rumpshaker* animals or between the different genotypes on the C57 background. This western blot was of low quality despite multiple attempts to produce a high quality blot and hence limited statistical inference can be made from the results.

Figure 3.19: Western blot analysis of MBP in C3H pellet fractions. There was significantly reduced MBP levels in the pellet fractions of the C3H *rumpshaker* (R,) mice compared to the wild-type (W).



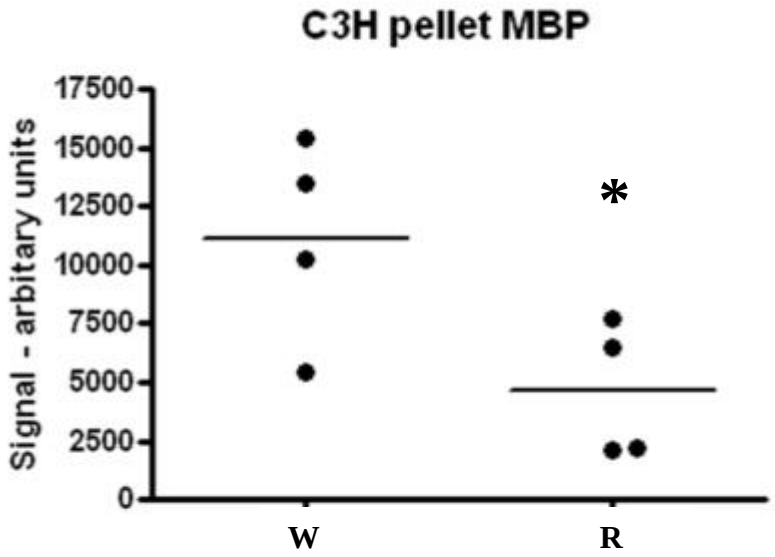
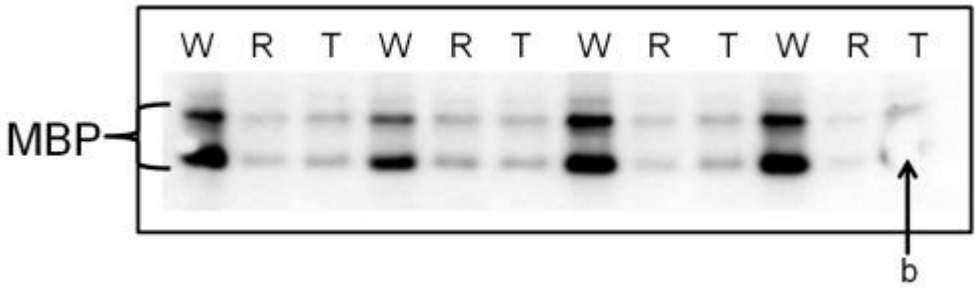


Figure 3.20: Western blot analysis of MBP in C57 pellet fractions. There were significantly lower MBP levels in pellet of the *rumpshaker* (R,) and *rumpshaker* transgene (T,) compared to the pellet of the C57 wild-type (W). There is an artefact in the gel (b) adjacent to the final T sample.



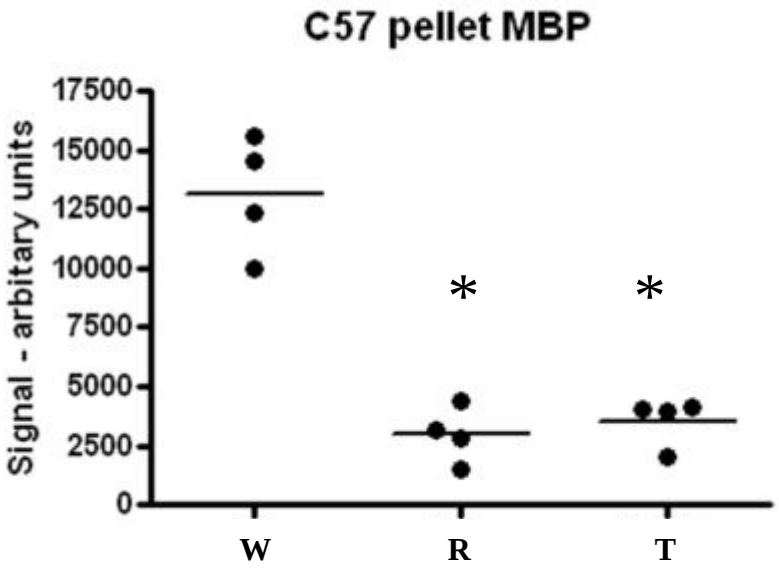
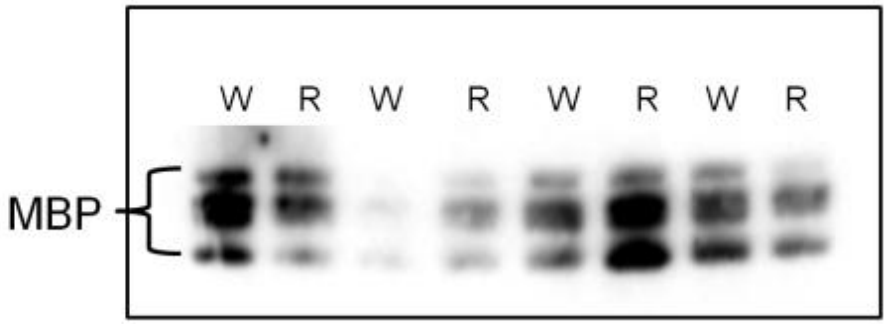


Figure 3.21: Western blot of MBP in C3H myelin fractions. There was no significant difference in MBP levels between C3H wild-type (W, WT) and *rumpshaker* (R, RSh) myelin. This western blot was of low quality despite multiple attempts to produce a high quality blot and hence limited statistical inference can be made from the results.



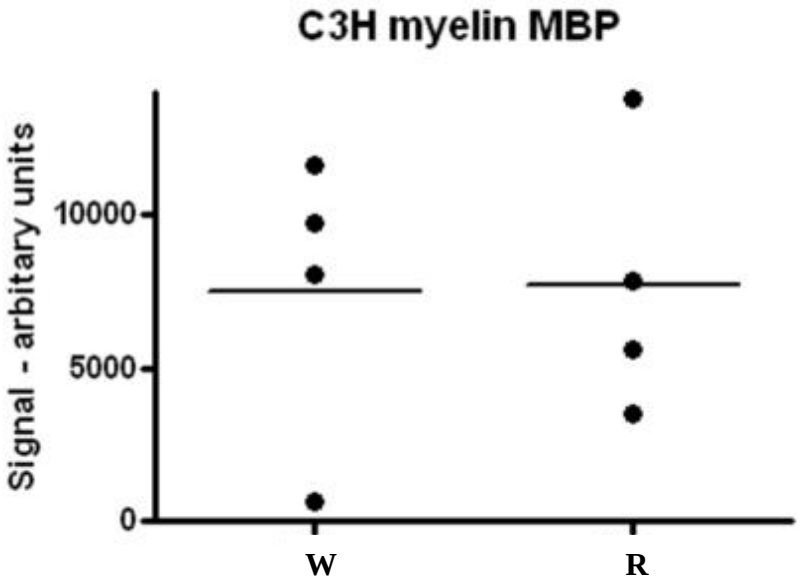
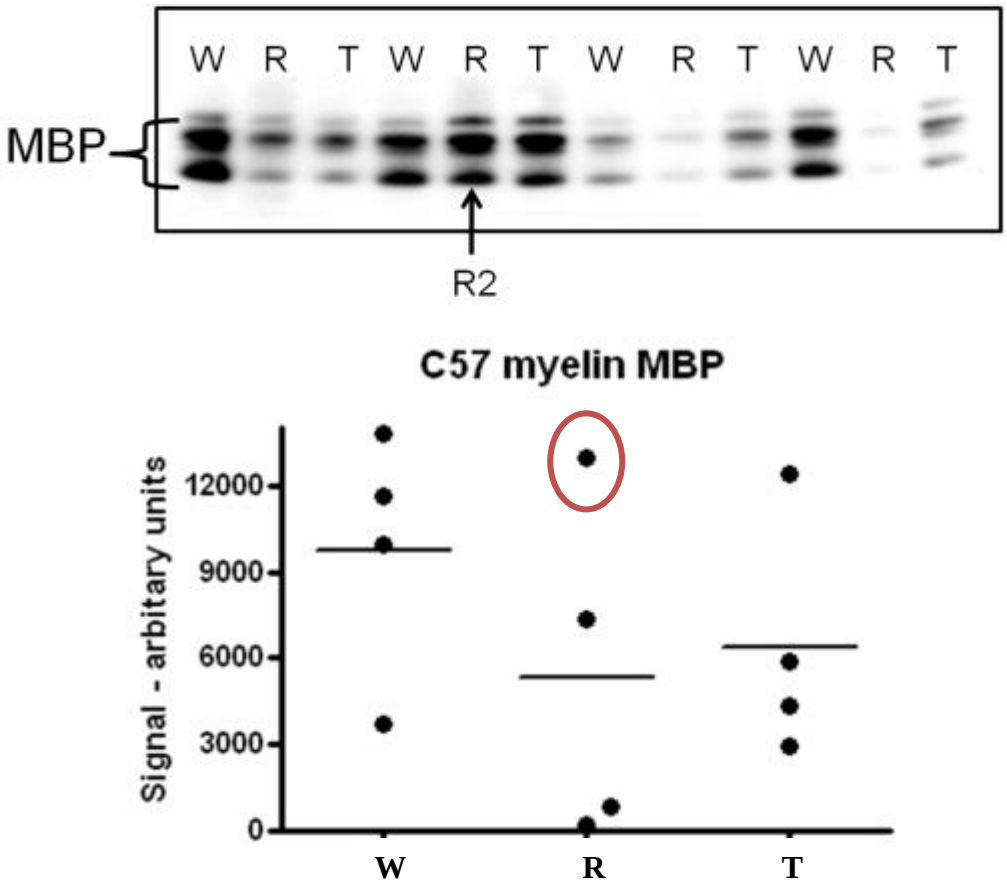


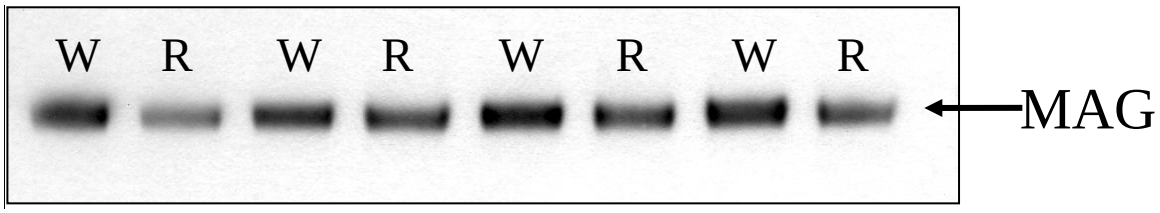
Figure 3.22: Western blot analysis of MBP in C3H myelin fractions. There was no significant difference in MBP levels in C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T). The high MBP levels in *rumpshaker* 2 (R2, red circle on graph) are noteworthy as this is the mouse that we suspect to be mis-genotyped.



3.4.8.2 MAG in C3H and C57 myelin samples

MAG was significantly reduced in the myelin of the C3H *rumpshaker* group compared to the myelin of the C3H wild-type group (P=0.0049). There were no significant differences in the levels of MAG in the myelin of any of the C57 groups. However there was a trend towards MAG being lower in the myelin of the *rumpshaker* and *rumpshaker* transgene mice (Figures 3.23 and 3.24).

Figure 3.23: Western blot analysis of myelin associated glycoprotein (MAG) in C3H myelin fractions. There was significantly reduced MAG in C3H *rumpshaker* (R) myelin compared to wild-type (W). and P<0.05



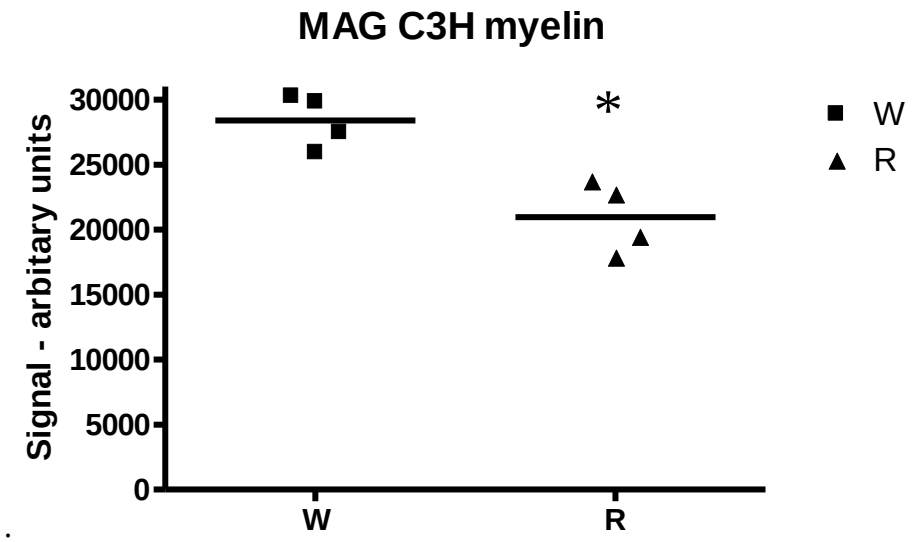


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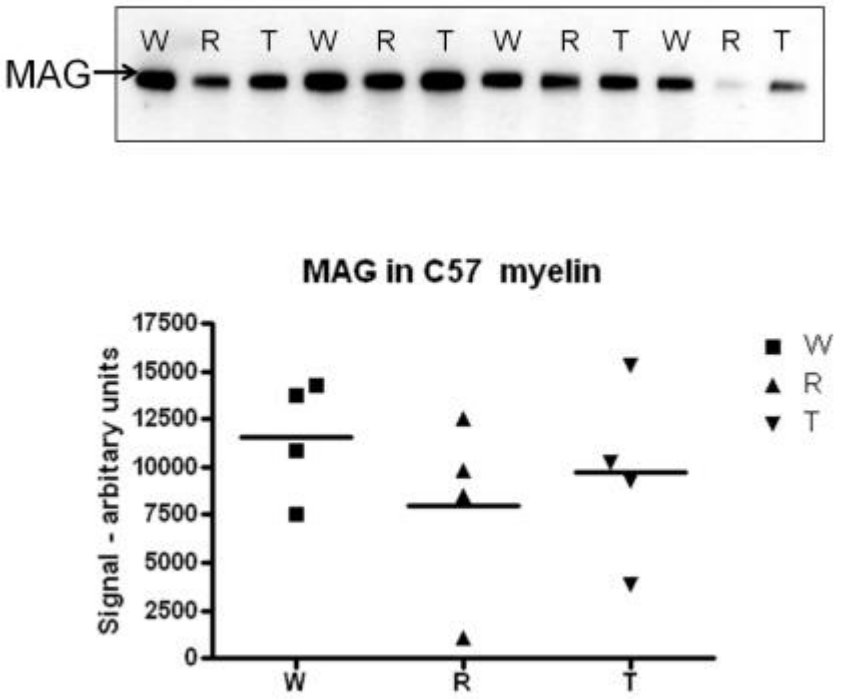
Figure 3.24: Western blot analysis of myelin associated glycoprotein (MAG) in C57 myelin fractions There was no significant difference in MAG levels in C57 wild-type (W), *rumpshaker* (R) and *rumpshaker* transgene (T).



3.4.9 Investigating the possibility of increased myelin contamination by non-myelin proteins in the rumpshaker samples.

As dysmyelenation occurs in *rumpshaker* mice, their central nervous tissue contains less myelin than that of wild-type mice. During myelin extraction, we questioned whether the lower volume of *rumpshaker* myelin would have a relatively greater proportion of contamination proteins from other fractions if this were to occur. As the pellet (membrane enriched) fraction may represent the source with the highest density of potential contamination of membrane proteins and may therefore influence the G-protein content of myelin, we explored ratio of two proteins that are present in this fraction but are not associated with G-proteins. Two proteins that are enriched in the pellet were chosen to measure in C3H *rumpshaker* and wild-type myelin; heat shock protein 90 (HSP90) which and aspartoacyclase (ASPA)).HSP90 is a molecular chaperone and present in the nuclear and

cytosolic fractions. ASPA is a membrane-associated enzyme which hydrolyses N-acetylaspartate and is important for normal white matter. Although ASPA has been reported to be present in myelin. (Wang *et al*., 2007)(Wang *et al*., 2007), it is enriched in pellet fractions. For the measurement of HSP90 and ASPA, the gels were loaded with 5µg and 2.5 µg samples of pellet and myelin (respectively). If myelin contamination had occurred to a greater degree in the *rumpshaker* mice, we would expect an increased myelin:pellet ratio of HSP90 and ASPA in the *rumpshaker* samples compared to the wild-type.

3.4.9.1 HSP and ASPA myelin;pellet ratio in C3H samples

There was no significant difference in the myelin;pellet ratio of either HSP90 or ASPA in C3H *rumpshaker* mice compared wild-type. There was a trend for the ratio to be slightly higher in the *rumpshaker*s but this was not statistically significant (Figures 3.25 and 3.26).

Figure 3.25: Western blot analysis of heat-shock protein 90 (HAP90) in C3H myelin and pellet fractions. There was no significant difference in myelin(M);pellet(P) ratio of HSP90 in C3H wild-type(W) and *rumpshaker* (R) myelin.

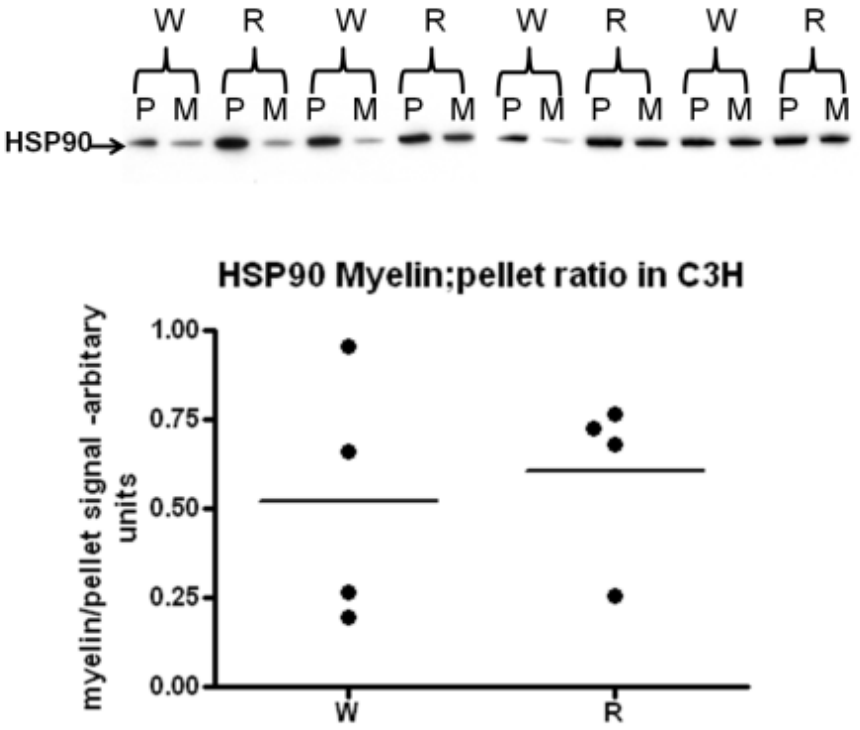
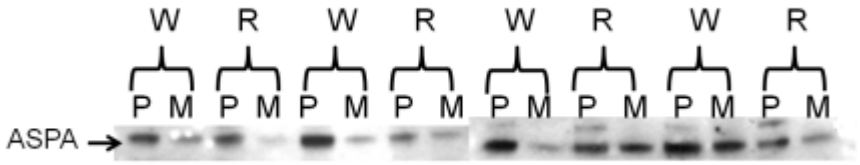
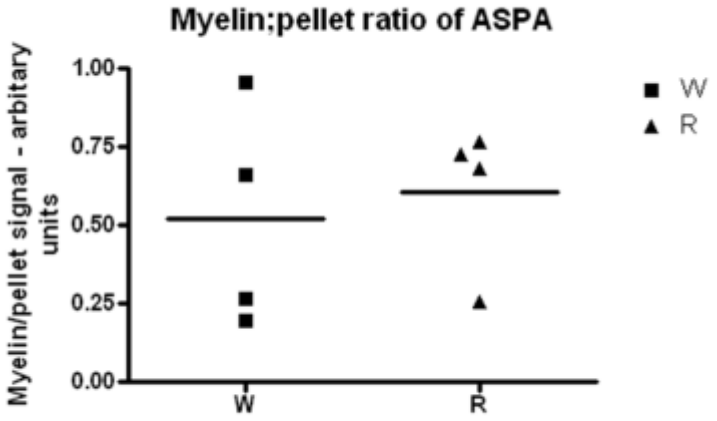


Figure 3.26: Western blot analysis of aspartocyclase (ASPA) in C3H myelin and pellet fractions. Western blot analysis showing no significant difference in the myelin(M);pellet(P) ratio of ASPA in C3H wild-type (W) and *rumpshaker* (R) myelin





Results in appendix:

Please see the appendix for western blots showing PLP and phosphoralyated MBP in C3H wild type and *rumpshaker* myelin and pellet fractions. Limited statistical inference could be made from these blots due to non-specific anti-body binding.

3.5 Discussion

3.5.1 Increased G proteins in C3H rumpshaker myelin

Our results show significantly increased levels of $G_{\alpha o}$ and $G_{\alpha q}$ in the myelin of C3H *rumpshaker* mice compared to that of matched wild-type controls. Ancillary investigations comparing the levels of MBP, MAG, HSP90 and ASPA indicate that is result is unlikely to be a methodological artefact associated with the extraction of the myelin from hypomyelinated *rumpshaker* compared to wildtype. These findings represent the first investigation into G proteins in *rumpshaker* myelin and provide a possible link in the chain of events between the *Plp* mutation and dysmyelination.

3.5.2 Link between Plp-1 mutation and rumpshaker phenotype

There have been detailed studies on pathological changes in the central nervous tissue of *rumpshaker* mice (Edgar *et al*., 2004a; Griffiths *et al* 1998;1990), the biochemical characteristics of PLP metabolism in these mice (McLaughlin 2006 and 2007) and the effect of PLP deficient myelin on the adjacent axon (Edgar *et al* 2004). It is also recognised that lack of PLP alone will not result in the *rumpshaker* phenotype (Klugman *et al*., 1997) , that MBP , unlike PLP, is absolutely essential for normal myelination (Readhead *et al*., 1987) and that MBP is reduced in *rumpshaker* mice (Al-saktawi *et al*., 2003). However, exactly how misfolded PLP results in hypomyelenation has eluded researchers to date (Woodward 2008). Our findings in this current study may provide the link between the missense *Plp-1* gene that is the known cause of hereditary spastic paraplegia, altered MBP function and subsequent dysmyelenation. The genetic background (C3H versus C57) upon which the *rumpshaker* mutation occurs has such a large impact on the phenotype of the mice (Al-saktawi *et al*., 2003; McLaughlin *et al.*, 2007). Hence a clear understanding this pathway from the *Plp-1* mutation to the end disease is vital for rational navigation of research and therapeutic efforts.

3.5.3 Connection between increased $G_{\alpha q}$ levels and reduced PIP2

To the author's knowledge, this is the first study that has measured G proteins in the myelin of mice with the *rumpshaker* mutation. The significant increase in $G_{\alpha q}$ levels in the C3H *rumpshaker* mice is confluent with the hypothesis that increased $G_{\alpha q}$ protein levels may be causing reduced PIP2 that was found in the pilot study or Dr McLaughlin (appendix). The work of Nawas *et al* (2009) highlighted the close electrostatic association between MBP and

PIP2, with dramatic reduction of MBP levels in the plasma membrane upon hydrolysis (artificially and by PLC) of membrane PIP2 and elevation of intracellular Ca^{2+}. Subsequent to their findings that elevated intracellular Ca2+ disrupts the membrane association of MBP, Nawas *et al* (2009) hypothesized that Ca2+ regulates the association of MBP with the plasma membrane in a Calmodulin dependent manner as has been observed with other basic unstructured proteins. Furthermore, the activation of protein kinase C via the PIP2 hydrolysis pathway is known result to in the phosphorylation of MBP (Vartanian *et al*., 1986) which may alter MBP properties and function.

3.5.4 Possible effects of increased $G_{\alpha o}$

In contrast to our findings with $G_{\alpha q}$, the significant increase in $G_{\alpha o}$ in the C3H *rumpshaker* myelin cannot be so readily hypothesised to be a causative factor for reduced PIP2 levels. $G_{\alpha o}$ is known to inhibit intracellular calcium release through breaking down DAG and also to prevent calcium influx into the cell. Furthermore it will reduce phosphorylation of proteins by inhibiting adenylate cyclise production (Jiang and Bajpayee 2009).Hence, any possible effects that $G_{\alpha o}$ might have on PIP2 are the opposite to those of $G_{\alpha o}$. Whilst $G_{\alpha o}$ is abundant in the central nervous system with essential functions relating to ion flux in neurons (Jiang *et al*., 1998), its function in myelin is unclear. $G_{\alpha o}$ is proposed by several studies to have a role neuron apoptosis in Alzheimer's disease (reviewed by Jiang and Bajpayee 2009). Perhaps there is also a apoptotic role for $G_{\alpha o}$ in the oligodendrocytes of *rumpshaker* mice. Apoptosis, contrary to the early report of (Griffiths *et al*., 1990), is now known to be a feature of *rumpshaker* central nervous tissue (Al-Saktawi *et al*., 2003). $G_{\alpha o}$ is sensitive to inhibition by pertussis toxin (Katada *et al*., 1982). Pertussis toxin inhibition of $G_{\alpha o}$ function (in primary oligodendrocyte cultures) may help clarify its role in myelin.

3.5.5 Reduced MAG levels in rumpshaker myelin

(Al-Saktawi *et al* (2003) reported levels of MAG in the myelin of C3H *rumpshaker* mouse to be 71% of that of C3H wild-type myelin. In the same study, myelin levels of MAG in C57 *rumpshaker* mice were 32% of wild-type levels. Reduced levels of MAG in *rumpshaker*s were also recorded in this current study. These reduced levels are consistent with the ideas of Griffiths *et al*. (1990), McLaughlin *et al*. (2006) and Barrie *et al*. (2010), suggesting that misfolded PLP may overload the endoplasmic reticulum and Golgi complex and interfere with synthesis of other proteins such as MAG. MAG normally resides in the myelin

membrane and it is known to be associated with maturation and maintenance of axons (Quarles , 2009). The objective of our measurement of MAG was to explore the possible impact of reduced myelin PLP in *rumpshaker* myelin on the relative quantities of other myelin proteins. This measurement of reduced MAG in C3H *rumpshaker*s in this study could be interpreted to suggest that reduced PLP in the *rumpshaker* was not causing relative increases in the proportions of other proteins subsequently relatively rather than absolutely elevated G proteins. Similarly, the equality of myelin;pellet ratios of HSP90 and ASPA (typically enriched in pellet fractions) suggests that any myelin contamination during preparation, whilst present at a low level, was equal in *rumpshaker* and wild-type samples. Together these findings support the contention that $G_{\alpha q}$ and $G_{\alpha o}$ are elevated in the myelin of C3H *rumpshaker* mice and are not an artifact arising from the alterations in the stiochiometric composition of proteins in myelin.

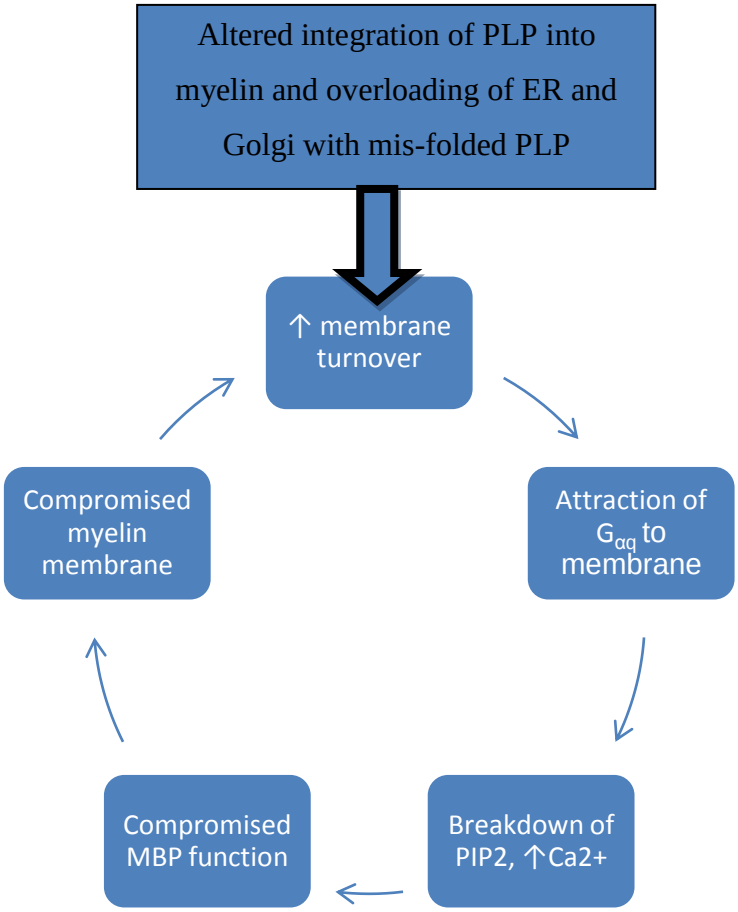
3.5.6 Hypothesis relating altered PLP to elevated G proteins and compromise of the myelin membrane

From these early findings of G protein elevations in myelin, the reasons for their elevation in the myelin of *rumpshaker*s can only be hypothesised. One possibility is, that misfolded PLP is being inappropriately integrated into the myelin membrane and that processing the misfolded PLP is overloading the endoplasmic reticulum and golgi complex (consistent with the swollen golgi and endoplasmic reticulm described in *rumpshaker*s by (Griffiths *et al*., 1990) and altered dynamics of PLP turnover (McLaughlin *et al*., 2006). This would alter lipid synthesis and increase myelin membrane turnover. Increased lipid turnover in the membrane would make it more likely to have non-lamellar areas which are known to attract G proteins including $G_{\alpha q}$ (Vögler *et al*., 2008). This mechanism would result in a "vicious circle". I.e. the integration of misfolded PLP into the myelin membrane is altered and the endoplasmic reticulum and Golgi systems are overloaded processing this misfolded PLP (mclaughlin 2007). This will increase membrane turnover, creating more active, non-lamellar areas in the membrane which attract G proteins. If $G_{\alpha q}$ is activated, this will cause increased PIP2 hydrolysis and subsequently further membrane breakdown and attraction of more G proteins, thus creating a cycle of membrane breakdown that is now independent of the misfolded PLP which instigated it (Figure 3.28).

Our findings with the C57 *rumpshaker*s were not conclusive. In the case of the pellet fractions, there was a trend for both $G_{\alpha q \text{ and }} G_{\alpha o}$ to be lower in the *rumpshaker* and *rumpshaker* transgene than in the wild-type. In the myelin fractions both of these proteins showed a trend towards elevation in the same genotypes. This contrasts to the trends towards increased in pellet $G_{\alpha q \text{ and }} G_{\alpha o}$ in the C3H *rumpshaker* and significant increases in myelin in $G_{\alpha q \text{ and }} G_{\alpha o}$. Pending further studies showing that this contrast in G protein behaviours in the different genetic backgrounds is significant, it could be postulated that G protein activity is somehow associated with ameliorating the severity of phenotype associated with the *rumpshaker* mutation. For example, accelerated hydrolysis of PIP2 may be important for co-ordinating MBP association with the myelin membrane. Until further G protein measurements and PIP2 analysis is conducted in the myelin of C57*rumpshaker*s, this will remain a purely speculative argument.

Our findings and hypotheses relating to G proteins could be exciting for two reasons. Firstly, they may allow us to better understand the pathway from *Plp-1* mutation. Secondly, G-protein coupled receptors lend themselves to therapeutic targeting, already being successfully targeted with many drugs, for example beta blockers (Milligan and Kostenis, 2006).

Figure 3.28 : Hypothesized connection between mis-folded PLP, increased $G_{\alpha q}$, PIP2 breakdown and compromised myelin membrane.



3.5.7 Further work and limitations of the current study

An initial step towards validating the above hypothesis would be to ascertain if the *rumpshaker* myelin contains more G protein because the oligodendrocyte is synthesising more or is it because more G protein is being attracted from the cell body to the myelin membrane. This could be addressed by performing primary cultures with *rumpshaker* and wild-type oligodendrocytes. Fluorescent anti-body studies could localise G-proteins and Western blotting could quantify the G proteins in both cultures. Another question is whether the G proteins that we are quantifying are in their active state. There are now commercial antibodies available for epitopes specific to activated G-proteins with which we could answer this question through further western blotting studies.

Whilst G protein levels in the C57 *rumpshaker* myelin showed a trend towards being reduced, our findings were not statistically significant. We suspect that one of the mice that we classified as "*rumpshaker*" ("*rumpshaker* 2") may in fact have been a mis-genotyped *rumpshaker* transgene, as the protein profile (particularly PLP) upon silverstaining of myelin from this mouse was more typical of a transgene. This possible misclassification may have confounded our low sample numbers. Little can be concluded from the trends for G protein changes in the myelin of the transgenic mice. Perhaps that the study should be extended to include more samples given the relatively high variation encountered in these experiments.

We hesitated to read too much significance into our C3H myelin MBP blots and our PLP blots due to heterogeneous quality of chemiluminescence and strong background luminescence on these blots. However changes in MBP and PLP in *rumpshaker* mice are well reported by previous authors (Barrie *et al*., 2010; McLaughlin *et al*., 2006).

The two unidentified protein bands of that were identified in the *rumpshaker* myelin samples upon silver staining were smaller in size than either $G_{\alpha o}$ or $G_{\alpha q}$ (both over 40kDal in size), eliminating the possibility that these were confounding our Western blot results by non-specific anti-body binding. It would be intriguing to identify these proteins that appear enriched upon silver staining in the *rumpshaker* myelin and a highly valid avenue for a mass spectrometric protein identification study.

In conclusion, this nascent study has identified significant increases in both $G_{\alpha q}$ and $G_{\alpha o}$ in the myelin of C3H *rumpshaker* mice. This, together with previous findings regarding G-protein membrane interactions, reduced PIP2 in *rumpshaker* myelin, MBP interactions with PIP2 and the essential role of MBP in myelination may facilitate the understanding of the pathway between PLP mutations and the spectrum of dysmyelination as well as adding to the understanding of other myelin-related diseases.

Chapter 4: General Conclusions

A broad spectrum of topics and techniques in molecular biology, epidemiology and cell biochemistry are documented in this thesis. Each of the projects is providing data to contribute to larger ongoing projects in the laboratories from where they originated.

In the first project, our findings confirmed that *lin-41* is present in *H. contortus* and that the 3′ UTR of this gene has potential *let-7* binding sites. The laboratory is now creating a bacterial plasmid construct using the *H. contortus* 3′ UTR sequence and a gene encoding green floursecent protein. If a transgenic *C. elegans* can be successfully created with this construct, *let-7* and *H. contortus lin-41* 3′ UTR interaction may be tested. This could then be used as a model with which to test methods for parasitic microRNA disruption. Validating such approaches in a trans-genic model would facilitate and expedite selection of the most viable microRNA disruption approach for testing in *H. contortus* itself, making most efficient use of resources and minimising the use of experimental sheep.

The second project endorses a web-based questionnaire as a powerful tool for analysis of British equine demography. Several demographic variables were highlighted where, after corroboration with multivariable analyses, should be focussed upon to improve vaccination coverage. The questionnaire is currently ongoing and will yield much valuable demographic information, providing more robust epidemiological artillery in the face of emerging and endemic equine infectious diseases.

Investigation of a fundamental concept; whether guanine nucleotide binding (G) proteins are altered in the *rumpshaker* model of Pelizeus Merzbacher Disease, was the focus of the third study. The identification of significant increases in $G_{\alpha q}$ and $G_{\alpha o}$ in the myelin of C3H *rumpshaker* mice has generated hypotheses about the pathway connecting the *Plp-1* mutation to the *rumpshaker* phenotype. It may also provide an answer for the important question of why the same mutation results in dramatically different phenotypes when imposed upon different genetic backgrounds. Altered G protein levels and previous findings regarding G-protein membrane interactions, reduced PIP2 in *rumpshaker* myelin, MBP interactions with PIP2 and the essential role of MBP in myelination may facilitate the understanding of the pathway between PLP mutations and the spectrum of dysmyelination. This may also add to the understanding of other myelin-related diseases.

Together, these projects have provided an invaluable opportunity to gain skills in a spectrum of scientific techniques. Whilst in diverse areas, each project addressed a disease challenge through a novel approach. There are increasing collaborations between scientists with different specialisms, as no one approach can address all aspects of a problem. There is a symbiotic relationship between epidemiology and the fundamental sciences. Hypotheses about disease can be generated through analysing field observations or questionnaires, as in project 2. These hypotheses can be explored in depth through the complementary techniques of molecular and cell biology, as in projects 1 and 3. Statistical techniques can then be used to make sense of laboratory findings and to put them in context. Finally epidemiological studies can be used to assess the effect of interventions arising from fundamental and translational research in the population. Hence this thesis represents a snapshot of novel ways to solve problems in 3 mutually complementary sciences.

Appendix

Project 1:

Figure A.1 Portion of H. contortus supercontig 0048734 which aligned with these sequences of Hc-lin-41 3′ untranslated region fragments amplified through 3′ rapid amplification of cDNA ends. The aligned portions are highlighted in yellow. It was this sequence that was searched for potential *let-7* binding sites.

CGTTTGGCCGTGCTGTCCTCTCGTGACACACCAGAAATGAAAGTATATGG
TACGGAGGGAAGTGTCGAGGGGATGTTCTGCCGCCCACAAGGAGTGAAGG
TTGATCCAGAGGGTCATATTCTTATTTGTGATTCTCGGAACAATCGCGTT
CAGGTTAGTTCTTTCATGCTTGTTCATCTGCTGTCTTTCTTAAACTGGTC
TGTCTGCAGGTATTTTCTGGAGATGACATGCGGTGCATCGCCGTCTTCGG
ACAATCATCCTCGTCTGGTGGTTTCGAGATGCCTGCTGAGCTACCTGCGC
CGTTCCGCTCTGTCGGGCCCACTCCCTTCCATGCCTCCACCACTCCTGCG
AGTGGCGTCGCTCTTGAAACCCCTACAGGTGGGCCTCGTCCATTGCTGGA
CCGCCCAACTGATGTGGCAATTGCCCCAGACGGTCGAATCTATGTGGTTG
TCGCTTCGCCTTCTAGCAACAGCTATTATGTTGCTGTTGTTCATCTCAAG
TGGAAATCTGGCTTCAAGCCAACATTTGTTTTGTGCTGTATCGCTAGGTG
AACATGTGCTATTTGTGCTTTGAGCCGCTGCGAGTCTCGCGTTCCCTTCA
GGCCTGCCTGCTCTTGTCATTTCCACATGTGATTCGATACGCTTCTTTTA
TTTCTCGTTGTTTGATTTGTTTCAAAGATTCACACAGAGTTTATTGTTCT
TCTCTCCACCGTAGTAACCACAGTCTCGTCCCCCTCCCTGCCCCATCCAC
ATATATTGCTCATATCACCTGGGCTTATCGCTCTTGTACACGATTCGCAT
GGTCTACCCAAGAGGTTCTGCTGGAGCTGTCTTTTCAAAACCTTTAATCT
CTCGCTCTCTCTCACACACGCTCTCTGTCCATGTTAATGTAACTGCTTCT
ACCTCCACCCTCGCATTCTAGCCGCTGACTAGCTGTTTCTTGTTACGGCG
ATCTACCTGCATACAACCGTCTTTACCTCAAACCATGCGAAACCTCGTCT
AATCCAGCCTCATCTCAGTTTTCGCATTCGAGACCTCCTTCGAGCATGTT
TTAGTCTATTAGCTAAAAACGCCTCATTTCCTTCAGCTCAGCGTACCCTA
CCGTTCTGTGAAAAGAAGGATTCCGGAACCGATGGGGAGATTCAGTCGTT
CGCTTATATTCCTTAGCTTTTGCCATGTCACTGTTTTAGTACCATCTTCG
TGTTCCGCGCCGCGGTTTTGTACAGTAGATTGCGAGGCCTCGCCCATTGT
CTTCAAGTCTAACTAGGGCCCCAATGCCCTTAACTGTGTGTCTCTATGTG
TGTGCACCTGCATTGTCTCGAATGCTCTCGAGGTAGACGATCGCCGTTTT
GATTTGTTTTAGTTACTTCCTTTCATGGATATCCAATTTCGCTTCTGACC
GCAGATTTCGTGATAGCGGTGTACATTGTTTTAACCGTTTTTATTACTGC
AGGGTATCGTTATGAGAGCCCAGTGAAAAGGAATACCAGTGAATATCCCG
GTTCGAATTGAATGTGTTTGAGAAAAGTTGAGCTATTTATTAGAGAGTAT
GTTTGTAAGCTAATCCTGGTGTTCAATGTTACGTTTTATCGCTTGTATTA
AAGTTCGTTTTGTTTTT

Appendix for project 2

Table A.1: Description of the proportions of horses vaccinated and unvaccinated against any of influenza, tetanus or herpes associated with demographic variables, including univariable odds ratios and likelihood ratio test P values of association with influenza vaccination. Blue and yellow highlighting denote decreased and increased odds (respectively) of a variable being associated with vaccination.

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated (%)	Vaccinated (%)	Coefficient	Standard error of coefficient	Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Type of premises									
Arable farm	173	4 (2.2)	178 (97.8)	0.90	0.26	0.368	0.335	1.59	0.58-4.36
Livery yard	991	18 (1.7)	1022 (98.3)	-0.43	0.25	0.001	<0.001	2.45	1.47-4.09
Livestock farm	405	20 (4.8)	396 (95.2)	0.46	0.52	0.087	0.101	0.65	0.4-1.06
Other premises	167	6 (3.5)	164 (96.5)	-0.08	0.47	0.911	0.912	0.95	0.41-2.2
Own premises	1025	39 (3.6)	1033 (96.4)	20.06	19620.70	0.523	0.57	0.88	0.59-1.31
Private yard	674	23 (3.3)	673 (96.7)	-0.13	0.20	0.903	0.902	1.03	0.65-1.64
Racing yard	16	0 (0)	14 (100)	-0.63	0.26	0.999	0.968	∞	0-∞
Rented pasture only	293	18 (5.7)	297 (94.3)	0.03	0.24	0.018	0.026	0.53	0.32-0.9
Riding school	149	5 (3.62)	133 (96.4)	-0.05	0.43	0.871	0.873	0.93	0.37-2.31
Region									
Midlands	372	8 (2.2)	364 (97.8)					REF 1	
East Anglia	427	15 (3.5)	412 (96.5)	-0.50	0.44	0.255	0.246	0.6	0.25-1.44
London	11	1 (9.1)	10 (90.9)	-1.52	1.11	0.171	0.247	0.22	0.03-1.93
North East England	367	10 (2.7)	357 (97.3)	-0.24	0.48	0.613	0.612	0.78	0.31-2.01
North West England	371	13 (3.5)	358 (96.5)	-0.50	0.46	0.27	0.263	0.61	0.25-1.48
Scotland	347	15 (4.3)	332 (95.7)	-0.72	0.44	0.105	0.096	0.49	0.2-1.16
South Central England	385	10 (2.6)	375 (97.4)	-0.19	0.48	0.687	0.686	0.82	0.32-2.11
South East England	304	8 (2.6)	296 (97.4)	-0.21	0.51	0.683	0.683	0.81	0.3-2.19
South West England	527	21 (4)	506 (96)	-0.64	0.42	0.131	0.117	0.53	0.23-1.21
Wales	174	10 (5.7)	164 (94.3)	-1.02	0.48	0.035	0.035	0.36	0.14-0.93
Activities									
Participates in at least 1 activity	3265	110(3.4)	3155(96.6)					REF 1	
No activities	20	1(5)	19(95)	-0.41	1.03	0.69	0.82	0.66	0.09-4.99
Travels to activities	2860	77(2.7)	2783(97.3)					REF 1	
Does not travel	425	34(8)	391(92)	-1.15	0.21	0.00	0.00	0.32	0.21-0.48
Affilliated competitions	999	12 (1)	987 (99)	1.31	0.31	<0.001	<0.001	3.72	2.04-6.81
Unaffilliated competitions	1870	25 (1)	1845 (99)	1.56	0.23	<0.001	<0.001	4.78	3.04-7.5
Hacking	3069	88 (3)	2981 (97)	1.40	0.25	<0.001	<0.001	4.04	2.49-6.53
Hunting	756	18 (2)	738 (98)	0.45	0.26	0.086	0.072	1.57	0.94-2.61
Breeding	569	17 (3)	552 (97)	0.15	0.27	0.57	0.564	1.16	0.69-1.97

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated (%)	Vaccinated (%)			Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Dressage	1699	18 (1)	1681 (99)	1.76	0.26	<0.001	<0.001	5.82	3.5-9.68
Trailblazers	359	4 (1)	355 (99)	1.21	0.51	0.018	0.004	3.37	1.23-9.2
Show jumping	1314	22 (2)	1292 (98)	1.02	0.24	<0.001	<0.001	2.78	1.73-4.45
Eventing	728	10 (1)	718 (99)	1.08	0.33	0.001	<0.001	2.95	1.53-5.68
Endurance	604	9 (1)	595 (99)	0.96	0.35	0.006	0.002	2.61	1.31-5.2
Driving	239	8 (3)	231 (97)	0.01	0.37	0.978	0.977	1.01	0.49-2.1
Western	156	6 (4)	150 (96)	-0.14	0.43	0.741	0.746	0.87	0.38-2.01
Showing	1326	26 (2)	1300 (98)	0.82	0.23	<0.001	<0.001	2.27	1.45-3.54
vaulting	44	0 (0)	44 (100)	20.07	11067.60	0.999	0.081	∞	0-∞
Point-to-point	97	2 (2)	95 (98)	0.52	0.72	0.471	0.434	1.68	0.41-6.91
Riding club.pony club	1318	14 (1)	1304 (99)	1.58	0.29	<0.001	<0.001	4.83	2.75-8.5
Lessons	2134	38 (2)	2096 (98)	1.32	0.20	<0.001	<0.001	3.74	2.51-5.57
Farrrier	2992	94 (3)	2898 (97)	0.64	0.27	0.018	0.027	1.9	1.12-3.23
Facilities	1785	22 (1)	1763 (99)	1.62	0.24	<0.001	<0.001	5.05	3.15-8.1
lessons	1835	30 (2)	1805 (98)	1.27	0.22	<0.001	<0.001	3.56	2.33-5.44
Other activities		10 (2)	486 (98)	0.60	0.34	0.072	0.052	1.83	0.95-3.52
Respondents' type of equestrian involvement									
Owner that rides	2940	82 (2.8)	2858 (97.2)	1.16	0.22	<0.001	<0.001	3.2	2.06-4.96
Owner non rider	220	18 (8.2)	202 (91.8)	-1.05	0.27	<0.001	<0.001	0.35	0.21-0.59
Non-owner that loans/rides	80	9 (11.3)	71 (88.8)	-1.35	0.37	<0.001	0.002	0.26	0.13-0.53
Professional	298	4 (1.3)	294 (98.7)	1.00	0.51	0.05	0.022	2.73	1-7.46
Livery yard proprietor	103	5 (4.9)	98 (95.1)	-0.39	0.47	0.403	0.428	0.68	0.27-1.69
Thoroughbred industry employee	25	0	25 (100)	20.06	14682.80	0.999	nc	∞	0-∞
Breeder	269	13 (4.8)	256 (95.2)	-0.41	0.30	0.171	0.192	0.66	0.37-1.2
Donkey owner	41	3 (7.3)	38 (92.7)	-0.83	0.61	0.172	0.221	0.44	0.13-1.44
Member travelling community	3	0	3 (100)	20.06	42385.60	1	nc	∞	0-∞
Other	490	17 (3.5)	473 (96.5)	0.12	0.46	0.799	0.796	1.13	0.45-2.8
Area next to premises									
Woodland	1283	31 (2.4)	1252 (97.6)	0.52	0.21	0.016	0.012	1.68	1.1-2.56
Grazing	2770	91 (3.3)	2679 (96.7)	0.17	0.25	0.491	0.498	1.19	0.73-1.95
Hay	1145	37 (3.2)	1108 (96.8)	0.07	0.20	0.732	0.731	1.07	0.72-1.6
Urban	177	7 (4)	170 (96)	-0.17	0.40	0.663	0.671	0.84	0.39-1.84
Semirural	728	21 (2.9)	707 (97.1)	0.21	0.25	0.404	0.394	1.23	0.76-1.99
Industrial	69	2 (2.9)	67 (97.1)	0.16	0.72	0.824	0.819	1.18	0.28-4.86
Running water	765	22 (2.9)	743 (97.1)	0.21	0.24	0.38	0.371	1.24	0.77-1.99
Standing water	383	11 (2.9)	372 (97.1)	0.19	0.32	0.56	0.55	1.21	0.64-2.27
Coastal	73	6 (8.2)	67 (91.8)	-0.97	0.44	0.026	0.048	0.38	0.16-0.89
Other	490	17 (3.5)	473 (96.5)	-0.03	0.27	0.904	0.905	0.97	0.57-1.64
International travel									
No international travel	3165	110 (3.5)	3055 (96.5)					REF 1	
International travel	120	1 (0.8)	119 (99.2)	1.46	1.01	0.149	0.062	4.28	0.59 - 30.95

Variable	Total Respondents (n= 3285 who answered vaccination questions)	Unvaccinated (%)	Vaccinated (%)			Wald P value	Likelihood ratio P value	Odds ratio	Odds ratio 95% confidence intervals
Imports									
Respondent doesn't import	2998	105 (3.5)	2893 (96.5)					REF 1	
Respondent imports	112	2 (1.8)	110 (98.2)	0.69	0.72	0.337	0.284	2	0.49-8.19
Respondent age-group				-0.09	0.36				
45 to 54 years old	919	25 (2.7)	894 (97.3)	-0.13	0.32		0.183	REF 1	
16 to 24 years old	404	12 (3)	392 (97)	-0.25	0.29	0.8	0.621	0.91	0.45-1.84
25 to 34 years old	519	16 (3.1)	503 (96.9)	-0.51	0.28	0.692	0.681	0.88	0.46-1.66
35 to 44 years old	721	25 (3.5)	696 (96.5)	-0.91	0.63	0.384	0.882	0.78	0.44-1.37
55 years or older	673	30 (4.5)	643 (95.5)			0.063	0.183	0.6	0.35-1.03
Under 16 years old	46	3 (6.5)	43 (93.5)			0.147	0.29	0.4	0.12-1.38
Respondent gender				-0.71	0.34				
Female	3127	101 (3.2)	3026 (96.8)					REF 1	
Male	158	10 (6.3)	148 (93.7)	-0.01	0.01	0.039	0.056	0.49	0.25-0.97
Horses owned									
Odds change per extra horse owned						0.337	0.383	0.99	0.97-1.01
				0.02	0.01				
Horses on premises									
Odds change per extra horse on premises						0.039	0.021	1.02	1-1.04
				-0.01	0.01				
Maximum number of hours travelled				0.20	0.10				
Odds change per extra hour travelled to horse care				0.23	0.05	0.157	0.216	0.99	0.97-1.01
Odds change per extra hour travelled to local events						0.057	0.008	1.22	0.99-1.49
Odds change per extra hour travelled to national						<0.001	<0.001	1.26	1.14-1.4
				0.90	0.26				
				-0.43	0.25				

Figure A.2: The variables which were associated with reduced odds of any vaccination (with 95% confidence intervals less than 1). The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1

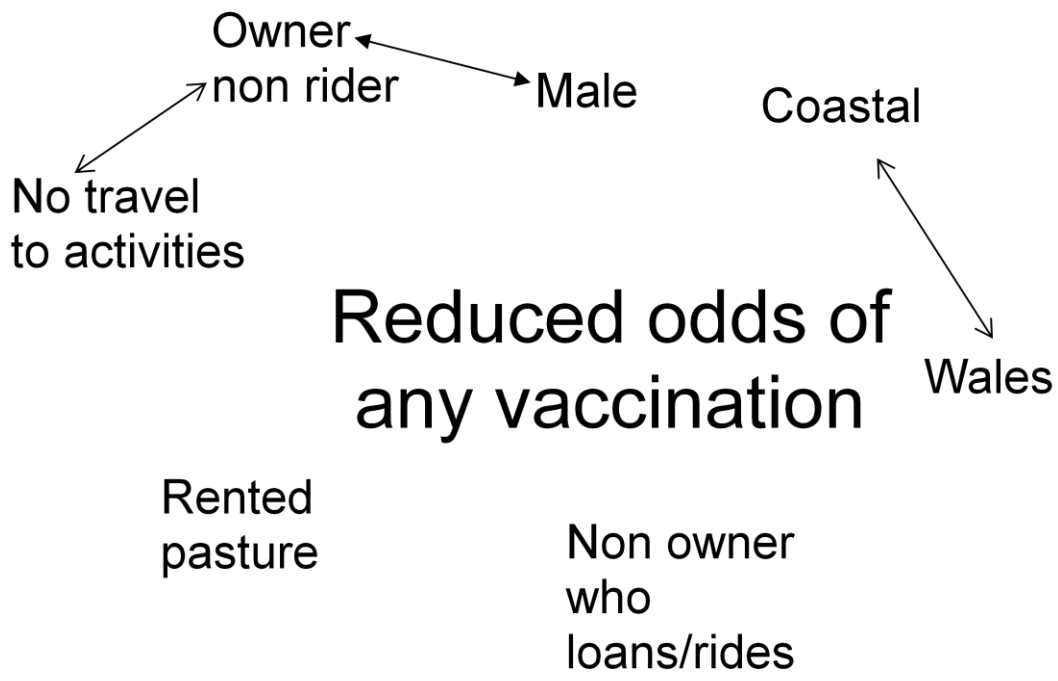


Figure A.3: The variables which were associated with increased odds of any vaccination (with 95% confidence intervals greater than 1). **The arrows between variables represent associations between them (the presence of one variable increasing the odds of the presence of the other with an odds ratio with 95% confidence intervals greater than 1). The activities highlighted in yellow are those associated with respondents that listed "Riding instructor/coach/professional professional" as describing their involvement with horses**

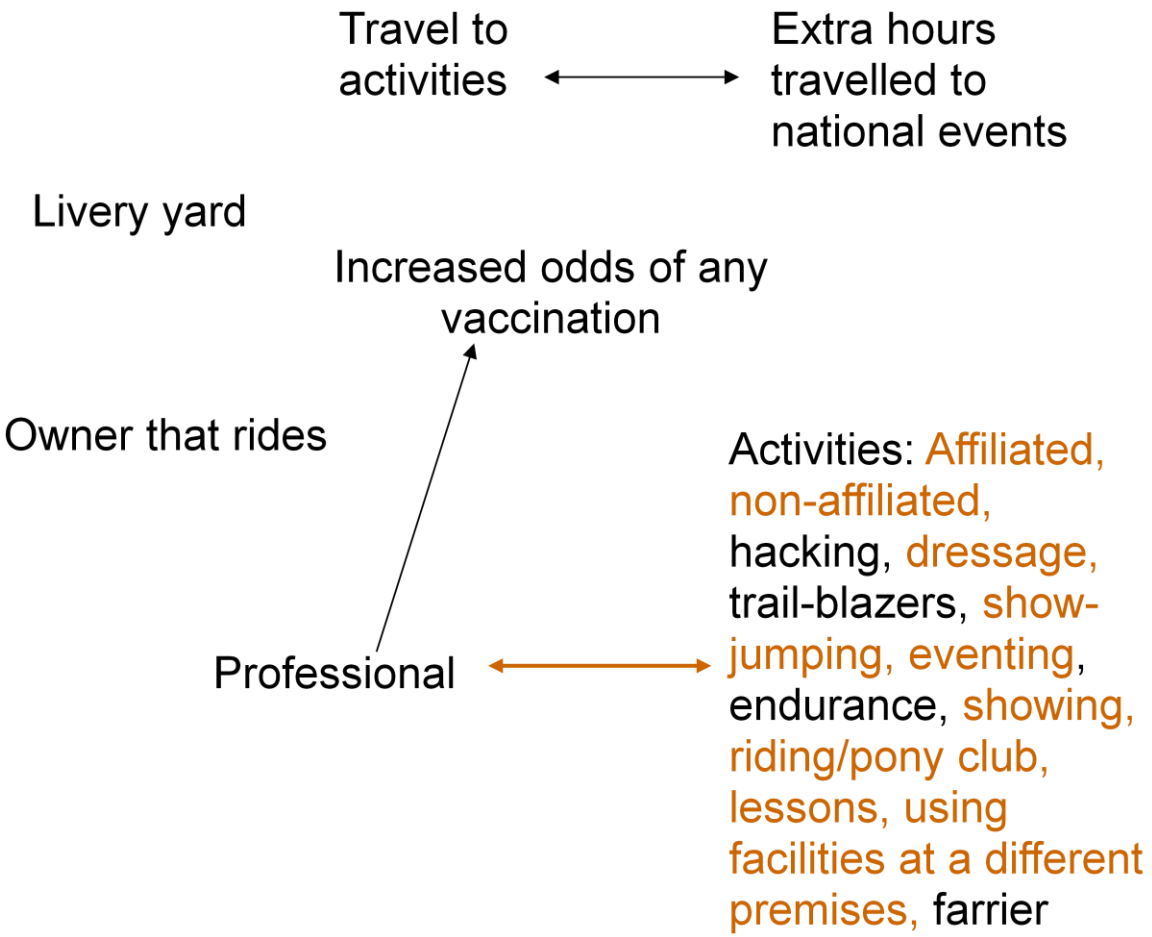


Table A.2: Associations between different demographic factors associated with influenza, tetanus, herpes and any vaccination.

Variable association	Coefficient	Standard error of coefficient	Wald P value	Log likelihood P value	Odds ratio	Odds ratio 95% Confidence Intervals
Own premises - Wales	0.83	0.14	<0.001	<0.001	2.29	1.73-3.02
Own Premises - South West England	0.40	0.09	<0.001	<0.001	1.50	1.26-1.78
Breeder - Wales	0.77	0.21	<0.001	<0.001	2.16	1.42-3.27
Breeder - Non rider	1.24	0.17	<0.001	<0.001	3.46	2.48-4.85
Breeder - Own premises	1.04	0.12	<0.001	<0.001	2.82	2.21-3.6
Breeder - Extra horses owned	0.14	0.01	<0.001	<0.001	1.15	1.13-1.18
>34 years old - Own premises	0.73	0.12	<0.001	<0.001	2.08	1.64-2.66
>34 years old - Breeder	0.67	0.23	0.004	<0.001	1.96	1.24-3.09
>34 years old - Extra horses	0.06	0.02	<0.001	<0.001	1.06	1.03-1.1
>34 years old - Non rider	1.07	0.30	<0.001	<0.001	2.91	1.61-5.25
Professional - International travel	1.37	0.22	<0.001	<0.001	3.93	2.57-6
Male - Owner non rider	0.58	0.25	0.019	0.03	1.79	1.19-2.91
<16 years old - Loans/rides non owner	1.27	0.53	0.018	0.02	3.55	1.25-10.12
Male - Extra horses	0.03	0.01	<0.001	<0.001	1.03	1.01-1.04
Male - Non rider	0.58	0.25	0.019	0.03	1.79	1.1-2.91
Scotland - Livestock farm	0.49	0.14	<0.001	<0.001	1.64	1.25-2.15
South West England - livestock farm	0.45	0.12	<0.001	<0.001	1.56	1.24-1.97
Driving - Pasture only	0.69	0.18	<0.001	<0.001	1.98	1.39-2.83
Driving - Extra horses	0.03	0.01	<0.001	<0.001	1.03	1.01-1.04
Driving - Breeding	0.67	0.19	0.001	<0.001	1.95	1.34-2.86
Travel to activities - International travel	1.81	0.59	0.002	0.00	6.11	1.94-19.32
Professional - travel to activities	0.75	0.23	0.001	<0.001	2.11	1.34-3.33
Professional - International travel	1.37	0.22	<0.001	<0.001	3.93	2.57-6
Professional - Affilliated events	0.94	0.12	<0.001	<0.001	2.56	2.02-3.23
Professional- Nonaffilliaated events	0.76	0.13	<0.001	<0.001	2.14	1.65-2.77
Professional-Hunting	0.81	0.12	<0.001	<0.001	2.25	1.77-2.87
Professional -Breeding	0.86	0.13	<0.001	<0.001	2.37	1.83-3.06
Professional - Dressage	1.19	0.14	<0.001	<0.001	3.29	2.51-4.3
Professional - Trailblazers	0.65	0.16	<0.001	<0.001	1.92	1.41-2.61
Professional - Show-jumping	0.85	0.12	<0.001	<0.001	2.34	1.84-2.96
Professional - Eventing	0.85	0.12	<0.001	<0.001	2.33	1.83-2.97
Professional - Showing	0.33	0.12	0.006	0.01	1.39	1.1-1.75
Professional - Vaulting	1.21	0.34	0.000	<0.001	3.35	1.73-6.48
Professional - Point-to-point	0.70	0.27	0.010	0.02	2.02	1.18-3.44
Professional - Riding/pony club	0.57	0.12	<0.001	<0.001	1.76	1.4-2.22
Professional - Lessons	0.87	0.15	<0.001	<0.001	2.39	1.79-3.19
Professional - Facilities at a different premises	0.87	0.15	<0.001	<0.001	2.39	1.79-3.19
Professional - Lessons at a different premises	0.80	0.13	<0.001	<0.001	2.22	1.72-2.87
Affilliated activities - private yard	0.19	0.09	0.036	1.2	1.01-	1.43
Affilliated - road hours to activities	0.017	0.004	<0.001	1.02	1.01-	1.03

Appendix for Project 3:

Figure A.4: The pilot study of Dr. Mark McLaughlin found significantly reduced levels of phosphotydlinositol 4,5 bisphosphate in the myelin of C3H *rumpshaker* mice compared to that of C3H wild-type mice. (n=4, studnets's T test P value = 0.0005).

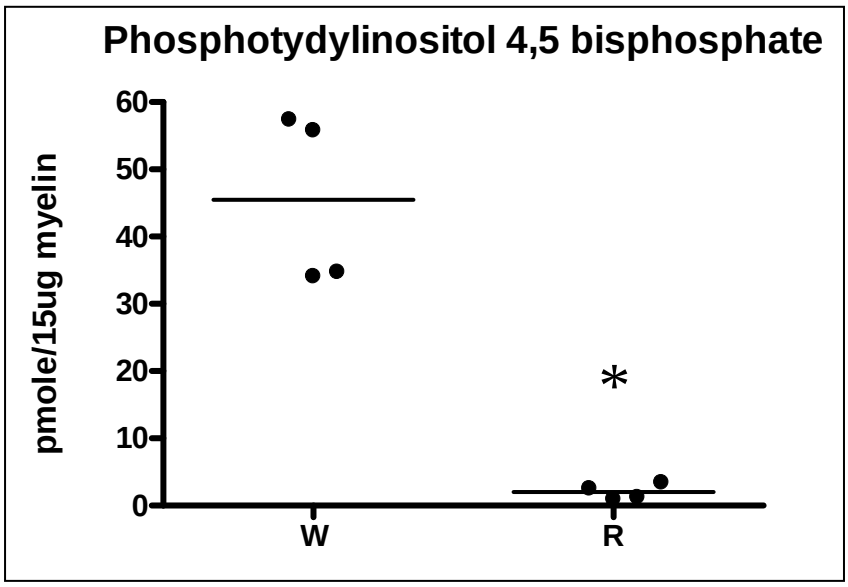
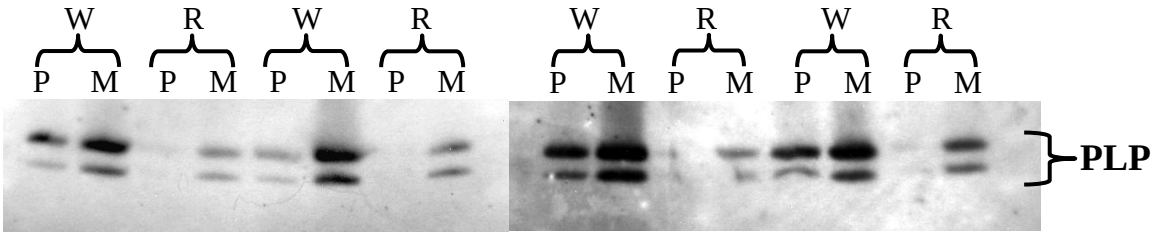
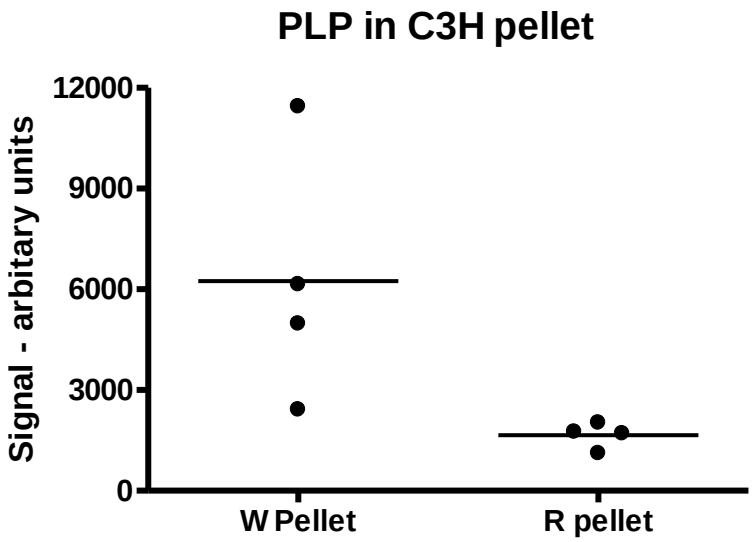


Figure A.5: Western blot analysis showing proteolipid protein (PLP) in C3H wild-type (W) and *rumpshaker* (R) pellet and myelin fractions. Quantitative analysis indicates significantly less plp in the nyelin of the *rumpshaker*. (P=0.043) Limited inference can be made from this result due to poor quality of the western blot.





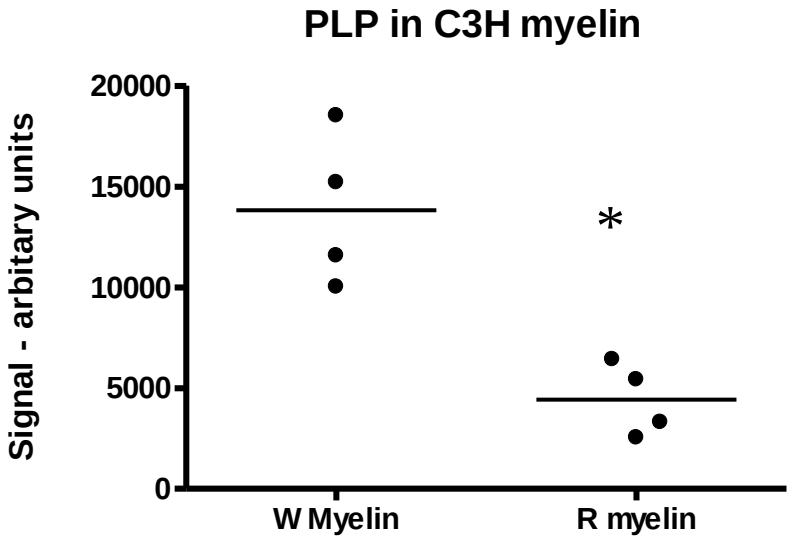
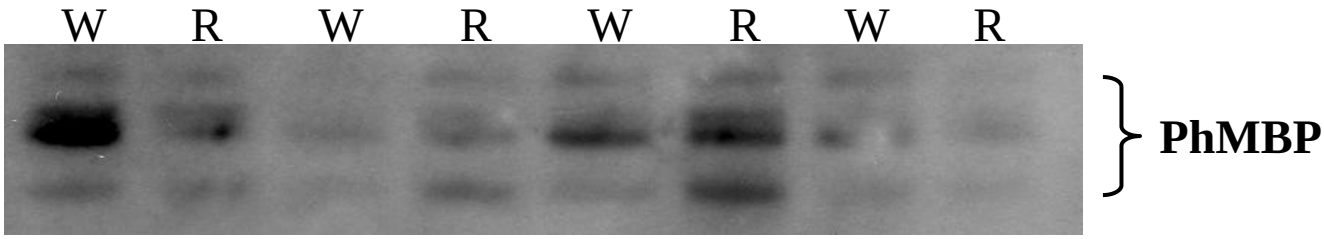


Figure A.6: Western blot analysis of phosphorylated MBP (PhMBP) in wild-type (W) and *rumpshaker* (R) myelin. The signal intensity was not measured due to hetrogenous blot quality.



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