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Title:
A mouse model of haemoplasma infection, in vitro cultivation of haemoplasmas and steps towards better diagnosis of and vaccination against haemoplasmosis

A mouse model of haemoplasma infection, *in vitro* cultivation of haemoplasmas and steps towards better diagnosis of and vaccination against haemoplasmosis

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A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy of the Faculty of Health Sciences, and the Bristol Veterinary School.

10th December 2019

Word count: seventy-nine thousand eight hundred and seventy-two words (excluding references, appendices and lists of contents)

ABSTRACT

The absence of a mouse model and successful *in vitro* cultivation systems for haemoplasmas have been a major hindrance in their research, impacting our understanding of haemoplasma pathobiology and delaying diagnostic means and vaccination strategies.

Rodent haemoplasmas were sourced from wild-caught field mice (*Apodemus* spp.) and 16S rRNA gene sequences used for identification and phylogenetic classification within the haemotropic *Mycoplasma* species. Novel rodent haemoplasmas were successfully passaged into immunocompetent, SPF Wistar rats and C57BL/6 mice. Haemoplasma-species specific quantitative polymerase chain reaction (qPCR) assays were developed for *Mycoplasma haemomuris* and *Eperythrozoon coccoides* and duplexed with an internal control qPCR to monitor infection in mice. Infection kinetics, partial protection from re-infection with the same isolate of *M. haemomuris*, and a lack of cross-protection resulting from previous *E. coccoides*-infection were described. Additionally, *M. haemomuris* was shown to induce anaemia during primary infection of mice.

To assess the use of *M. haemomuris*-induced haemoplasmosis of mice as a surrogate model for feline infection with *Mycoplasma haemofelis*, a cytokine signature of naïve infection and protection from re-infection was identified using a bead-based cytokine immunoassay. Principal component analysis identified a Th-17-driven immune response during naïve infection.

Using the mouse model of haemoplasma infection to generate viable *M. haemomuris* organisms, *in vitro* cultivation was attempted in liquid culture and mice used to assess *in vitro* survival of *M. haemomuris* by inoculating infected culture media into naïve mice and subsequent monitoring for infection by qPCR. *Mycoplasma haemomuris* remained viable for 8 consecutive days of *in vitro* cultivation and replenishment of culture media with fresh mouse blood resulted in *in vitro* growth of *M. haemomuris* for four consecutive days.

The newly established mouse model of haemoplasma infection and first ever successful *in vitro* cultivation of a haemoplasma will help further haemoplasma research in a range of veterinary relevant species.

DEDICATION AND ACKNOWLEDGEMENTS

This thesis would not have been possible without the ongoing support of my supervisors, Prof. Séverine Tasker and Dr. Chris Helps who actively supported my professional and personal growth over the past 3 ½ years and showed me by example how to conduct well-constructed research and stay motivated throughout countless setbacks. I am especially indebted to Prof. Séverine Tasker, who has been supportive of all my professional ambitions and served as a role model and mentor inside academia and in life in general. I have learned more from her and her family than I could ever give them credit for here. She has taught me not only how to be a good scientist but a good person and not only take ownership of my research project but my personal wellbeing as well.

Special thanks also go to the remainder of my supervision team, Dr. Daryll Hill for his insights in bioinformatics, Prof. Toby Knowles for sparking enthusiasm for statistics in me and Prof. Mick Bailey for training my scientific mind through various discussions and also for taking over the main supervision during the final stages of my PhD.

I am grateful to the generous funding of The Langford Trust who never questioned my abilities throughout many ambitious projects, including those that are not presented within the current thesis. I am also extremely thankful to Zoetis Animal Health who generously funded the laboratory rodents that comprised the foundation of this thesis. Special thanks also go to Prof. Ludwig Hoelzle from the University of Hohenheim for providing *M. suis* samples. Within the University of Bristol, I would like to thank to Dr. Ash Toye and Dr. Tim Satchwell from the School of Biochemistry for not only providing murine cell lines but also training me in their successful maintenance.

I also would like to thank the members of the postgraduate office at Bristol Veterinary School, especially Miss Louisa Mitchard, Miss Amy Thomas, Dr. Tom Chisnall, Dr. Oré Francis, Dr. Emily Barker and Dr. Emily Porter, who enriched the past years by sharing helpful insights, laughter and a plentiful amount of cake. Scientific research can be a lonely place, but you have taught me that it does not have to be. Heartfelt thanks also go to Sharon Holt for sourcing countless reagents and long-lost items of equipment and to Dr. Doug Wilson for sharing his knowledge on protein expression that sadly did not make it into the final manuscript. I would also like to thank Dr. Joe Roe and the technicians of the Animal Services Unit for their support throughout the various stages of the mouse projects.

Of course, all of this would not have been possible without the support of my parents who paved the way for my academic career not only through words of encouragement and financial support but also through backing various extravagant endeavours that made a PhD seem relatively normal. I would also like to thank my sister Katrin, for keeping me grounded and looking up to me as a colleague as I do to her. In the completion of the final manuscript, nobody has been more important than my partner Richard, who was unafraid of getting to know me during the second half of my PhD and has continuously made me see what I was capable of.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Reearch Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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1 INTRODUCTION

1.1 PHYLOGENY OF HAEMOTROPIC MYCOPLASMAS

1.1.1 First identification and phylogenetical classification

Haemoplasmas (haemotropic mycoplasmas) are small (0.3 to 0.9µm), wall-less bacteria that show erythrocyte tropism in various mammalian host species and are able to induce clinical disease (mainly anaemia) or remain asymptomatic for prolonged periods of time (1). They were first reported by Mayer et.al. in 1921 as 'bacteria-like' organisms causing anaemia in rats and subsequently named *Bartonella muris*. Similar small (< 1µm) bacteria, adhering to erythrocytes and causing anaemia, were later found in splenectomised mice (2) and dogs (3) and non-splenectomised sheep (4), cattle (5), cats (6) and pigs (7). Initially, these organisms were identified as *Bartonella* spp. and *Eperythrozoon* spp. and the *Bartonella*-named organisms later transferred to *Haemobartonella* gen. nov. to allow differentiation from other disease-causing *Bartonella* genus bacteria (8). Bacteria of the genus *Haemobartonella* were differentiated from those of the genus *Eperythrozoon* by differences in their microscopic appearance, as *Eperythrozoon* organisms could be seen unattached to erythrocytes on blood smears and as 'ring forms' (1, 9).

1.1.2 Phylogenetic re-classification and ongoing disputes

Haemobartonella and *Eperythrozoon* species were first classified within the family of *Anaplasmataceae* in the *Rickettsiales* order. However, they differed from *Anaplasmataceae* by their lack of a cell wall and lack of motility organelles, their inability to form inclusion bodies, their intra-erythrocyte replication and their intrinsic resistance to penicillin (10). These differences led to a revision of species names and transfer of several species previously named *Haemobartonella* (*Haemobartonella muris, Haemobartonella felis, Eperythrozoon suis* and *Eperythrozoon wenyonii*) to new genera within the order *Mycoplasmatales* (class *Mollicutes*), family *Mycoplasmataceae* (1), despite significant dissimilarity to other mycoplasmas in this family (11). These transfers led to all haemoplasmas being given *Candidatus* designation, which is typically reserved for incompletely described taxa, but the status was maintained due to the inability to grow haemoplasmas *in vitro*. However, the *Candidatus* designation was later dropped for *Mycoplasma haemomuris, Mycoplasma haemofelis, Mycoplasma suis* and *Mycoplasma wenyonii* (12), as these species previously existed. *Mycoplasma ovis*, formerly named *Eperythrozoon ovis* (4) was later transferred to the genus *Mycoplasma* as well (13) whilst *Eperythrozoon coccoides* retained its name (14, 15). Ongoing phylogenetic controversy exists due to two genera (*Mycoplasma* and *Eperythrozoon*) being used to describe very closely related organisms. The controversy is unlikely to be resolved until whole genome sequences and *in vitro* cultivation systems can be derived for all species affected (11) and may potentially require formation of a novel haemoplasma genus (16, 17).

1.1.3 Phylogenetic characteristics of haemoplasmas

With the availability of rapid deoxyribonucleic acid (DNA) sequencing, gene sequences that are present in all prokaryotes have been used to describe haemoplasma phylogenetic relationships. The 16S rRNA gene is highly conserved amongst prokaryotes and occurs at least once in each bacterial genome. Thus, sequence identities of the 16S rRNA gene can be used to measure taxonomic diversity. Based on sequence identities, thresholds have been proposed for separation of species, genus, family, order, class and phylum (18); 86.2% for family, 94.5% for genus (18), and 97% to 99% for species resolution (19) with 98.65% being a widely used threshold and identities above 97% warranting further investigation (20). These thresholds contribute to ongoing disputes over the

phylogenetic classification of haemoplasmas as they have between 74% and 77% 16S rRNA sequence identity to the reference strain of the *Mycoplasma* genus, leading to some authors rejecting their classification within this genus (21).

Phylogenetic trees can be produced using 16S rRNA gene sequence. Traditionally the neighbour-joining method was used to cluster phylogenetic identities based on the number of DNA base substitutions; the number is then reflected in the length of a branch (22). With more advanced computing, these simple clustering algorithms have been replaced with evolutionary models (accounting for reversals or convergence) that work by repeated sampling of data (bootstrapping) to create a phylogenetic tree based on the maximum likelihood (relating to the number of iterations where certain branches have formed) method (23). Based on 16S rRNA gene-derived phylogenetic trees, and irrespective of phylogenetic methodology used, the haemoplasmas cluster with the pneumoniae-group of the *Mycoplasma* genus, but reside within their own clade. They do split into two subgroups that have been named after their feline representatives, *M. haemofelis* and '*Candidatus* Mycoplasma haemominutum' although the porcine pathogen *M. suis* is also used to refer to the latter group of haemoplasmas (1, 24-26). Phylogenetic trees based on the RNase P RNA gene (*rnpB*) have shown similar relationships (17).

However, the 16S rRNA gene (and even more so *rnpB*) is small, comprising around 1,500 base pairs and thus has limited positions available for base substitutions (27). *In situ* hybridisation of genomic DNA to DNA of a reference species (DNA-DNA hybridisation; DDH) has been used to resolve species above the 97% threshold and is still highly recommended to resolve species that share more than 98.2% 16S rRNA gene sequence identity. The DDH methodology however is time-consuming and only available for organisms that can be grown in pure culture to obtain the required genomic DNA (28). Alternatively, multi-locus sequence analysis (MLSA) has been established as a phylogenetic approach to increase the available number of mutations by combining individually sequenced housekeeping genes (23, 29). Whole-genome phylogeny is another method that can be applied if whole genome sequences are available, but typically requires culturable organisms to generate the material required and is not widely established (30). Hicks, Barker (16) applied MLSA to haemoplasma phylogeny using fragments of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heat-shock protein 70 (DNAK); this study also suggested that haemoplasmas should indeed form their own genus. The phylogeny showed that the very closely related species of *M. haemofelis* and *M. haemocanis* (16) shared > 99% of their 16S rRNA gene (24), and an average of 94.9% of their rnpB gene sequences (31). However, the number of gene sequences per putative species for phylogenetic studies was relatively small and comprised one to 12 (*M. haemofelis* only) sequences per species (16, 17, 26). Thus, expected variations within the same species might have been underestimated, as has been described for other bacteria (32).

1.1.4 Haemoplasmas identified so far

Haemoplasma species identified from their respective hosts and sufficiently different from one another to be considered a separate species (as discussed in 1.1.3) are listed in **Table 1**.

Current species* name	Host animal(s)
Mycoplasma haemomuris (12) *Eperythrozoon coccoides*** (14) '*Mycoplasma haemomuris*-like haemoplasma' (33)	Murine rodents (rats and mice)
'Mycoplasma haemomuris-like haemoplasma' #2 (34, 35) '*Candidatus* Mycoplasma turicensis'-like haemoplasma (35, 36)	Other muroid rodents (e.g. hamsters, harvest mice and voles)
Mycoplasma haemofelis (1) '*Candidatus* Mycoplasma haemominitum' (37) '*Candidatus* Mycoplasma turicensis' (38) '*Candidatus* Mycoplasma haematoparvum-like haemoplasma' (39)	Cats (including wild felids)
Mycoplasma haemocanis (24) '*Candidatus* Mycoplasma haematoparvum' (40)	Dogs (including foxes and wolves)
Mycoplasma suis (12) *Eperythrozoon parvum*** (7)	Pigs (including wild boars)
Mycoplasma ovis (13)	Sheep and goats
'Candidatus Mycoplasma haemovis' (41) '*Candidatus* Mycoplasma erythrocervae' (42) *Mycoplasma wenyonii* (43)	
'*Candidatus* Mycoplasma haemolamae' (24)	Camelids
'*Candidatus* Mycoplasma haematorandirangferis' (44) '*Candidatus* Mycoplasma haemocervae' (45) '*Candidatus* Mycoplasma erythrocervae' (45) *'Mycoplasma ovis*-like *haemoplasma'* (46) *'Mycoplasma wenyonii*-like *haemoplasma'* (47)	Deer
Mycoplasma wenyonii (12) '*Candidatus* Mycoplasma haemobos' **** (48)	Cattle (including water buffalos)
'Mycoplasma haemocanis-like *haemoplasma' #2 (49)*	Black bears
'*Candidatus* Mycoplasma kahaneii' (50) '*Candidatus* Mycoplasma aoti' (51) '*Candidatus* Mycoplasma haemomacaque' (52) '*Candidatus* Mycoplasma haemomacaque'-like haemoplasma (53) '*Candidatus* Mycoplasma kahaneii'-like haemoplasma (54)	Primates
'*Candidatus* Mycoplasma haemodidelphidis' (24)	Opossum
'*Candidatus* Mycoplasma haemomeles' (55)	Badger
'*Candidatus* Mycoplasma haemozalophi' (56)	Sea lions
'*Candidatus* Mycoplasma haemohominis'-like haemoplasmas (57)	Bats
'*M. coccoides*-like haemoplasma' (58)	Capybara
'*Candidatus* Mycoplasma haemohominis' (59) *Mycoplasma haemofelis*-like haemoplasma (60) *Mycoplasma suis*-like haemoplasma (61)	Humans
'*Candidatus* Mycoplasma haemominutum'-like haemoplasma (62)	Chelonians
'Mycoplasma suis-like haemoplasma' and '*Mycopplasma wenyonii*-like' haemoplasma (63)	Non-mammalian host*** (ambrosia beetle)

Table 1 *Haemoplasma species identified so far. *Species that have been assigned taxonomic names are listed in full and other isolates identified by their closest, named relative (<98.2 % 16S rRNA gene sequence identity). **Eperythrozoon coccoides and Eperythrozoon parvum have been placed within the genus Mycoplasma by various authors. *** Haemoplasmas have been found in blood-sucking arthropods such as ticks and fleas but are not listed separately as haemoplasmas were identical with haemoplasmas found in mammals. **** Synonym: 'Candidatus Mycoplasma haemobovis' (64).*

Additional diverse haemoplasma species and strains, depending on 16S rRNA gene identity thresholds, have been reported in a number of wild carnivores such as weasels, stone martens, badgers, wolves and foxes (65). Multiple novel haemoplasma species have also been found in bears (66), non-human primates (67), rodents (35, 68), bats (69) and raccoons (70), but not included in **Table 1** due to lacking discriminatory power over related species and/or strains. A haemoplasma organism has also been found in horses, sharing 98.2% of its 16S rRNA gene sequence with '*Ca.* M. haemobos' (71).

1.1.5 Characteristics of haemoplasma genomes

Nine haemoplasma whole genomes sequences have been reported, as listed in **Table 2**. Haemoplasma genomes comprise a single circular chromosome and utilise the UGA-codon to encode tryptophan, which is common to the Mycoplasmas (72, 73). The genome of '*Ca.* M. haemominutum' could not be closed, likely due to secondary structure surrounding the origin of replication (74), and the genome of '*Ca.* M. haemobos' is published in draft form of 18 overlapping sequence reads (contigs) and has not yet been fully annotated (73).

Species name	Genome size (base pairs)	GC content (%)	Putative proteins encoded	Percentage of paralogous gene sequences (%)
Mycoplasma haemofelis (75, 76)	1,147,259	38.9	1580 - 1593	71.5 – 72.4
'*Candidatus* Mycoplasma haemobos' (73)	935,638	30.5	n/a	n/a
Mycoplasma haemocanis (77)	919,992	35.0	1173	63.0
Mycoplasma haemolamae (78)	756,845	39.3	925	49.1
Mycoplasma suis (79, 80)	742,431	31.1	794 - 845	37.4 – 42.8
Mycoplasma ovis (81)	702,511	31.7	801	32.4
Mycoplasma wenyonii (82)	650,228	33.9	652	40.3
'*Candidatus* Mycoplasma haemominutum' (74)	513,880	35.5	547	24.5
Mycoplasma parvum (83)	564,395	27.0	581	26.0

Table 2 *Haemoplasma species with whole genome sequences available for genomic analysis. The genome of 'Candidatus Mycoplasma haemominutum' has been published as discontinuous and the genome of 'Candidatus Mycoplasma haemobos' (synonum to 'Candidatus Mycoplasma haemobovis') is currently published as a draft genome sequence, comprising 18 contigs.*

Their genomes comprise 547 to 1,593 coding sequences and within them, a high percentage of paralogous repeat sequences (homologous genes arising through gene duplication, considered a driving factor in bacterial evolution) (72). These sequences are organised in families of up to 800 sequences (in *M. haemofelis*) and are correlated with genome size. Most of them are hypothetical proteins of unknown function (72) but are thought to play a role in immune evasion as some of them have been demonstrated to be immunogenic in *M. suis* (84) and *M. haemofelis* (85), which are the two most studied haemoplasmas. Such paralogous gene families have been shown to contribute to phase variation of antigenic proteins in other *Mycoplasma* spp. (86-88). Phase variation could be

associated with the cycling of haemoplasma copies in blood over time seen in *M. haemofelis*-infected cats (89, 90).

Immunogenic haemoplasma proteins have been identified in *M. suis* (84, 91-93) and *M. haemofelis* (94); these have been protein-folding machinery proteins (e.g. DNAK and its cofactor heat-shock-protein-24) or glycolysis pathway proteins (e.g. GAPDH, enolase) or other core metabolic enzymes (e.g. inorganic pyrophosphatase). The finding that haemoplasma core metabolic enzymes can be involved in pathogenicity and hence become immunogenic in their hosts highlights the multi-use of these proteins by haemoplasmas (called 'moonlighting') and is described for other *Mycoplasma* spp. (95, 96). However, the number of haemoplasma proteins that have an orthologous (genes arisen from a common ancestor in multiple species) protein annotated within the Clusters of Orthologous Groups (COG) database (http://www.ncbi.nlm.nih.gov/COG) is low, only 295 to 319, except for the related *M. haemofelis* and *M. haemocanis* species, whose genomes encode about three times as many COG-identified proteins (72). No data on COG-identified proteins are available for '*Ca.* M. haemobos' (73). The low level of similarity to known proteins and the unculturable status of the haemoplasmas is a significant problem for *in silico* prediction of haemoplasma proteins and immunogenic epitopes for use vaccines or serological assays.

1.2 HAEMOPLASMA INFECTION AND CLINICAL HAEMOPLASMOSIS IN MAMMALS

Haemoplasma infection has been detected in a variety of livestock and companion animal species as summarised in **Table 1**. Haemoplasma infection is not always associated with clinical disease; this is largely dependent on the haemoplasma species and sometimes the host immune system (97). The pathogenesis of haemoplasma infection and development of clinical haemoplasmosis have been studied mostly in cats for feline haemoplasmas, including data on detection, prevalence and risk factors (89, 98-105), genomics (16, 25, 26, 74, 75, 106), proteomics and immunology (94, 107-110). Similar studies exist in pigs, including *M. suis* detection and prevalence (111, 112), pathogenesis (113-116), proteomics and vaccine candidates (84, 117-121) whilst other haemoplasma species are less studied. As this thesis is aimed to further knowledge on feline haemoplasma infection and prevention of clinical haemoplasmosis in cats, data on feline haemoplasma species will be discussed, followed by translational aspects of porcine haemoplasma infection and clinical haemoplasmosis caused by *M. suis.*

1.2.1 Feline clinical haemoplasmosis and haemoplasma infection

In cats, clinical haemoplasmosis was previously referred to as 'feline infectious anaemia' (122), but to avoid confusion with other infectious causes of anaemia in cats, this term is no longer used. For the purpose of this thesis, the term 'haemoplasmosis' alone refers to haemoplasma infection, with or without clinical disease.

Three haemoplasma species are known to infect cats, with *M. haemofelis* the most likely to cause clinical disease such as pyrexia and (sometimes fatal) anaemia (123). The species '*Ca*. M. haemominutum' is rarely associated with clinical disease but, similarly to '*Ca*. M. turicencis', may cause reductions in erythrocyte count and haemoglobin concentration (90). '*Candidatus* M. turicensis' has been reported to cause more severe clinical haemoplasmosis in immunocompromised cats, but can also cause disease in immunocompetent cats (38, 124). Clinical haemoplasmosis manifests as lethargy, pyrexia, inappetence, weight loss and anaemia signs such as pallor, pica, haemic heart murmur and tachypnoea. Hepatosplenomegaly is reported, and icterus is uncommon (90). Two more haemoplasma species have been reported in cats; '*Ca*. M. haematoparvum-like haemoplasma' that has yet to be described fully or shown to be associated with disease (39, 125),

and *M. haemocanis* that was reported to be successfully passaged through cats to then infect dogs, although clinical disease was not seen in the *M. haemocanis*-inoculated cats (126).

Prevalences of the three most common feline haemoplasmas, as determined by quantitative polymerase chain reaction (qPCR), have been reported from various geographical locations and range from 0.4 – 46.6% for *M. haemofelis*, 10 – 32.1% for '*Ca*. M. haemominitum' and 0.4 – 26% for '*Ca.* M. turicensis' as reviewed by Tasker (123). Higher prevalences of haemoplasma infection are generally reported for warmer climates (39, 102, 127, 128) and can sometimes coincide with higher prevalences of arthropod vectors within a country (102).

The finding of infections with all three haemoplasma species in cats without clinical suspicion of haemoplasmosis shows the presence of a carrier state that likely presents a source of infection for naïve cats (106, 129, 130); the carrier status most commonly occurs with '*Ca*. M. haemominutum' and this may contribute to its high prevalence (131).

Antibiotic therapy for feline clinical haemoplasmosis typically comprises tetracyclines or flouroquinolones, and these treatments can result in rapid resolution of clinical signs caused by *M. haemofelis* following a two to four-week course of oral antibiotics, however *M. haemofelis* infection is not usually cleared from blood (89, 103, 130, 132-134). Clearance of *M. haemofelis* infection has only been recently demonstrated with one protocol; oral doxycycline for four weeks followed by oral marbofloxacin for two weeks if bacteraemia (positive qPCR results) persisted (135). Treatment of '*Ca*. M. haemominutum' with oral marbofloxacin is also reported to reduce bacterial loads (136), but due to the lack of clinical signs with this species it remains less studied. Infection with '*Ca*. M. turicensis' was reported to respond to either oral doxycycline for two weeks or consecutive doxycycline then marbofloxacin administration (124), but in contrast to *M. haemofelis*-associated clinical haemoplasmosis, clinical signs can resolve without therapy (102).

The anaemia of clinical haemoplasmosis is thought to be mediated through damage to the erythrocyte membrane as haemoplasmas attach themselves and subsequently induce haemolysis and removal of infected erythrocytes in the spleen (123). One study demonstrated that cold-reactive (4°C) autoantibodies appeared as early as 8 days post-inoculation with *M. haemofelis*, coinciding with the development of anaemia, but warm-reactive (37°C) autoantibodies were only found 22 days post-inoculation, by which time a regenerative anaemia was present (90). Additionally, another study only demonstrated autoagglutination of erythrocytes when sera of *M. haemofelis*-infected cats and infected erythrocytes were combined, or when sera of *M. haemofelis*-infected cats and erythrocytes treated with neuraminidase (to alter their surface) were combined (137). This suggested that antibodies are directed at haemoplasma epitopes or altered erythrocyte surface epitopes but not to normal erythrocytes. Autoantibodies on erythrocytes were not demonstrated in '*Ca*. M. haemominutum' nor '*Ca*. M. turicensis' infection, although this may be due to insensitive methods used (Coombs' testing) for their detection in the study (90).

Due to limitations in the feline host size and hence blood volume (for e.g. collection of large volumes of haemoplasma-infected blood) and ethical considerations, studies targeting the feline host-pathogen interaction and haemoplasma protein expression have been greatly hindered by the lack of an *in vitro* cultivation system to study at least some of these interactions outside of the natural feline host. No vaccination studies have been reported although passive immunisation (using convalescent plasma from *M. haemofelis*-infected cats) showed no protection when transferred to naïve cats prior to inoculation with *M. haemofelis* (138).

1.2.2 Porcine clinical haemoplasmosis and haemoplasma infection

Porcine clinical haemoplasmosis (previously porcine eperythrozoonosis) has been described as a febrile disease of swine with anaemia, icterus and haemagglutination that could be controlled through oxytetracycline treatment and hygienic measures, including control of lice (139). Two species of haemoplasmas have been described to infect pigs; *M. suis* and *Eperythrozoon parvum*, however the latter has no known pathogenicity (83). *Mycoplasma suis* is found in pigs worldwide and has also been found in wild boars in Europe (113). Acute infection causes potentially fatal haemolytic anaemia but chronic infection is linked to poor growth and reproductive performance and poses a significant economic risk to the swine industry (112). Prevalence determined by qPCR ranges from 13.0% to 18.2% but less data are available than for feline haemoplasma infection (113).

In contrast to feline clinical haemoplasmosis (typically caused by *M. haemofelis*), *M. suis* has been reported to invade erythrocytes (115), cause endothelial damage and result in hypoglycaemia due to excess glucose consumption in the host (113). Two *M. suis* immunogenic adhesion proteins have been identified that represent 'moonlighting' proteins of the glycolysis pathway; MSG1 (GAPDH) and α-enolase (91, 93) and *M. suis* DNAK has also been shown to be expressed during acute infection, and it elicits an immune response in pigs (119). Adhesion of *M. suis* to porcine erythrocytes alters their membranes and induces eryptosis (programmed cell death of erythrocytes and subsequent removal by the spleen) and limits clinical disease and anaemia in low-virulence strains that do not result in sudden intravascular haemolysis (140). Anaemia has been associated with warm-reactive erythrocytic IgG autoantibodies (141, 142). *Mycoplasma suis* also appears to contribute to a coagulopathy through its close association with endothelial cells, and *M. suis* appears to form biofilm-like colonies with erythrocytes and the endothelium, contributing to immune-evasion (114). Experimental vaccination attempts with recombinant *M. suis* MSG1 resulted in measurable immune responses but were not protective against *M. suis* challenge (120).

Due to their large size and availability for use in research, and the ability to be able to repeatedly sample them for quite large volumes of blood, pigs can be more amenable to haemoplasma research than cats. However, ethical considerations still exist with their use. Additionally, our understanding of their host-pathogen interaction is still very limited by the lack of an *in vitro* cultivation system for porcine haemoplasmas that would allow us to study endothelial and erythrocyte targeting outside of the host (113, 117).

1.2.3 Haemoplasma infection and clinical haemoplasmosis in other companion and livestock animals

In dogs *M. haemocanis*, and even more so '*Ca*. M. haematoparvum', appear only able to cause clinical anaemia in immunosuppressed (e.g. splenectomised) animals and hence have not received much scientific interest beyond eradication from kennel-raised research colonies (143) or individual case reports (144, 145). Prevalence, as detected by qPCR, varies from 0.8 to 26.2% for *M. haemocanis* (146, 147) and 0.3 to 33.4 % for '*Ca*. M. haematoparvum (148, 149), with higher prevalences generally in warmer climates. The latter supports the hypothesis that arthropod vectors, especially *Rhipicephalus sanguineus* (brown dog tick), are involved in transmission (150). However, Aktas and Ozubek (147) were unable to detect canine haemoplasma DNA in unfed *Rhipicephalus* spp. ticks collected from the same habitat as a Turkish shelter dog population with a high haemoplasma prevalence. A similar survey of partially-fed ticks collected from dogs in the UK also did not reveal any canine haemoplasma DNA (151), so the role of ticks in transmission remains uncertain.

Clinical haemoplasmosis caused by *M. ovis* has been described in anaemic lambs in association with poor growth or even death (152), but *M. ovis* may not cause clinical signs in adult sheep (153). Caprine clinical disease appears to be more severe but less commonly investigated (13). Hornok, Meli (41) found concurrent haemoplasma infection with *M. ovis* and a novel haemoplasma '*Ca.* M. haemovis' in sheep with haemolytic anaemia. Haemoplasma prevalence, as determined by qPCR, in sheep ranges from 14.1% to 45.8% with similar percentages (6.2% to 44.1%) reported in goats (42, 43, 154, 155). However, prevalence data are limited to selected geographical locations. Research is currently focused on sheep as clinical haemoplasmosis can be fatal and is difficult to clear with tetracyclines (42, 43, 156, 157). Subclinical infection with one or more haemoplasmas, including '*Ca*. M. erythrocervae', is also an emerging problem in sheep used for medical research as infection has been shown to alter haematological parameters and erythrocyte osmotic fragility (42). Bovine clinical haemoplasmosis, caused by *M. wenyonii* and the more recently discovered '*Ca*. M. haemobos', is associated with clinical signs such as depression, pyrexia and reduced milk production (158, 159). Regional qPCR prevalences range from 4.9 to 38.5% for *M. wenyonii* (160, 161) and 38.1 to 64.2% for '*Ca*. M. haemobos' (160, 162). The pathophysiology of bovine clinical haemoplasmosis is poorly understood as some studies only report fatal disease in conjunction with *Anaplasma* spp. infection (163, 164). Bovine haemoplasmas are considered an emerging pathogen in Northern Europe (161, 165).

In camelids, limited data are available but experimental infection with '*Ca*. M. haemolamae' has resulted in mild, transient anaemia and pyrexia (166). Prevalence data is limited to selected regions and ranges from 9.26 to 19.3% in clinically healthy South American llamas and alpacas (167). Meli, Kaufmann (168) reported 11 of 24 clinically healthy Swiss alpacas to be '*Ca*. M. haemolamae' infected, and Crosse, Ayling (169) found 38 of 131 UK alpacas were infected; however both of these studies were described following individual deaths in the group and hence prevalences may be higher than expected.

In contrast to other haemoplasmas, with the potential exception of *M. suis* for which vertical transmission had been suspected in earlier studies (139, 170), transplacental transmission of haemoplasmas has only been demonstrated for '*Ca.* M. haemolamae' (171, 172) and the bovine haemoplasmas *M. wenyonii* and 'Ca. M. haemobos' (165, 173, 174).

1.2.4 Zoonotic potential of haemoplasmas

Haemoplasma infection of humans is limited to small studies and case reports of immunocompromised individuals or individuals with reported close contact to animals (52). However, human haemoplasmas represent emerging zoonotic pathogens, especially in view of their association with warmer climates and respective availability of arthropod vectors (52). Santos and Santos (60) detected a *M. haemofelis*-like haemoplasma in a human immunodeficiency virus (HIV)-infected patient and Steer and Tasker (59) reported a novel haemoplasma named '*Candidatus* Mycoplasma haemohominis', related to rodent haemoplasmas, in a potentially immunocompromised patient. Sykes, Lindsay (175) reported two strains of an *M. ovis*-like haemoplasma in a veterinarian co-infected with *Bartonella henselae*, the same co-pathogen that was also found in a veterinarian infected with a '*Ca.* M. haematoparvum'-like haemoplasma (176). Maggi and Compton (52) confirmed haemoplasma infection in individuals with close contact to animals, in 9 of 193 individuals that self-reported chronic illness. Eight of these nine infections were due to the *M. ovis*-like haemoplasma and one due to a '*Ca*. M. haematoparvum'-like haemoplasma. Following up the potential association of chronic disease with haemoplasma infection, the same study found two infections (one *M. ovis*-like and one *E. coccoides*-like) in a cohort of 296 patients recruited from a rheumatologist's case load (52).

A *M. suis*-like haemoplasma was found in an anaemic patient in China (177) and in 32 of 65 veterinarians and farm workers on Chinese pig farms with a high *M. suis* prevalence. However, the genotypes were not identical (98% sequence identity on a partial 16S rRNA gene fragment) so interspecies pig-human transmission could not be proven (61).

1.3 DETECTION OF HAEMOPLASMA INFECTION

1.3.1 Blood smear examination

Haemoplasmas traditionally were diagnosed based on light microscopic examination of blood smears stained with a Romanowsky-type stain. When visible they appeared as small (< 1µm) pleomorphic rods or cocci that were found either alone or in short chains on the erythrocyte surface (7, 126, 178). However, differentiation from artefacts or other erythrocyte inclusion bodies (e.g. Howell-Jolly bodies (179)) was difficult and false positive results were common (180).

Following the development of more sensitive diagnostic molecular techniques, the sensitivity of blood smear examination was found to low, down to 11.1% in one study (128) and 21.3% (111) and 37.4% (165) in others.

1.3.2 Polymerase chain reaction (PCR)-based detection methods

Polymerase chain reaction allows the amplification of specific sections of DNA (181). The method has dramatically improved diagnostic sensitivity and specificity for pathogen detection, especially pathogens that are unculturable or grow slowly.

Typically, PCR assays target hypervariable regions in otherwise conserved genes, e.g. the 16S rRNA gene, as their sequences are readily available and do not require whole genomes or knowledge of the specific bacterial strain (182). Several PCR assays have been developed for haemoplasmas, including *M. haemofelis* (130), *M. haemomuris* (183) and *M. suis* (184). Primer design to less variable regions, that are still specific to haemoplasma genomes, allows the development of generic haemoplasma PCR assays to detect previously unknown haemoplasma species (185).

Quantitative PCR (qPCR) employs exponential amplification, like conventional PCR, but includes the use of an additional DNA sequence (probe) to bind to the amplified region; this probe fluoresces proportionally to the amount of amplified DNA, allowing quantification of the original bacterial DNA within the sample (186). As probes can be designed to fluoresce at different wavelengths, these assays can be duplexed with a host housekeeping gene to confirm the absence of PCR inhibitors and the presence of amplifiable DNA in the sample, as currently used for some feline and canine haemoplasma qPCRs (100, 104, 187). More recently, qPCR assays targeting protein-coding genes have been designed for bovine haemoplasmas; but the use of these is potentially limited to the individual strain, as protein-coding sequences are typically not included in sequencing surveys and hence intra-species variability of the assay target regions cannot readily be assessed (161, 188).

Quantitative PCR detection allows monitoring of infection kinetics following experimental infection with haemoplasmas. Following experimental intravenous infection, *M. haemofelis* is detectable by qPCR after one to two days, after which rapid growth and cycling bacteraemia (> 4 log-fold increases/decreases) occur (103, 189). This cycling is not seen in '*Ca*. M. haemominutum' (136) nor '*Ca*. M. turicensis' infection (124). Peak bacteraemia is typically reached after two weeks and coincides with the onset of clinical anaemia (90) with *M. haemofelis*. Infection with '*Ca*. M. haemominutum' reaches similar levels of bacteraemia as *M. haemofelis* infection (approximately 1.0E+09 genome copies per ml blood) whilst '*Ca*. M. turicensis' infection has much lower bacteraemia (1.0E+06 genome copies per ml blood) (90). Acute infection can be followed by a carrier

state in *M. haemofelis*, '*Ca*. M. haemominutum' (90) and sometimes '*Ca*. M. turicensis' infection (129), which can last for months. A low-dose subcutaneous infection model has been described for *M. haemofelis* by Baumann, Novacco (189) that resulted in a longer lag-phase after inoculation; cats became qPCR positive 19 days following inoculation compared to five days following intravenous inoculation (90).

The infection kinetics of *M. suis* are less investigated but rapid increases of bacterial loads following experimental intramuscular infection (up to 1.0E+11 genome copies per ml blood) have been described. Peak bacteraemia coincides with the onset of clinical signs at 15 days following inoculation and levels remain high for months (120).

Recently, Cohen, Shemesh (34) described the infection kinetics for a novel *M. haemomuris*-like haemoplasma in gerbils with persistent infection occurring for 700 days (the study duration); infection was first detectable at 15 days post-inoculation and peaked 10 to 30 days later at 1.0E+09 genome copies per ml blood, followed by a gradual decline (> 6 log-fold decrease over three months). Anaemia and potential cycling of bacterial loads were not assessed (34).

1.3.3 Other stain-based methods

In other blood-borne pathogens, such as *Plasmodium* spp., combined DNA-stains and mitochondrial membrane potential stains allow specific and sensitive detection of parasitaemia using flow cytometry to differentiate parasitized erythrocytes from inclusion bodies or other blood cells (190). Sánchez-Pérez, Brown (191) reported the use of flow cytometry using a DNA stain to monitor response to therapy in *M. haemofelis* clinical haemoplasmosis. However, as haemoplasmas lack organelles (such as mitochondria that would allow the use of specific stains), erythrocytic Howell-Jolly bodies and DNA remnants cannot be easily differentiated from haemoplasmas, as is possible for *Plasmodium* spp. using the mitochondrial potential stain. Other DNA stains alone, such as acridine orange, do not have increased diagnostic sensitivity when compared to light microscopy using Romanovsky-type stains (111, 165).

1.3.4 Serology

Serological detection assays require the production of specific antibodies against pathogen proteins or the production of immunogenic protein epitopes. In contrast to direct pathogen detection, serology is limited by the need for seroconversion and formation of specific antibodies, and positive results can occur due to previous exposure or in chronic carriers in the absence of overt bacteraemia. Techniques, such as enzyme-linked immunosorbent assays (ELISAs), have been adapted for patient-side use to allow rapid detection of infection (192). Crude haemoplasma antigen preparations have been used previously to develop serological assays but had poor sensitivity and specificity (193-195). Reliable haemoplasma serological assays require knowledge of immunogenic haemoplasma proteins, which is hindered by the absence of an *in vitro* cultivation system and available whole genome sequences.

Hoelzle, Hoelzle (142) identified eight immunogenic *M. suis* proteins using sera from acutely infected pigs, three of which were consistently immunoreactive over the course of infection. The group went on to develop sensitive ELISAs for experimental *M. suis* detection using *M. suis* DNAK and GAPDH proteins, but broader diagnostic use was limited by cross-reactive and autoreactive antibodies during acute clinical haemoplasmosis (91, 121).

Guimaraes, Santos (84) developed a multiplex microbead immunoassay, coupling three recombinantly expressed *M. suis* immunogenic proteins (heat-shock-protein-24, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase and one member of the paralogous repeat family) to

microscopic beads for flow cytometric detection. The resulting assay resulted in apparent increased sensitivity (78.43%), compared to qPCR (9.8%), however cross-reactivity to other pathogens was not assessed.

Immunogenic proteins have also been identified for *M. haemofelis* by Barker, Helps (94), who identified *M. haemofelis* DNAK to be highly immunogenic and developed an ELISA-based assay that had increased sensitivity (31.1%) over qPCR (9.8%). The ELISA was later shown to cross-react with antibodies against '*Ca.* M. turicensis' infection (129), but cross-reactivity to other pathogens was limiting its clinical use (94). Similarly, *M. haemofelis* GAPDH, and a number of other unidentified protein/protein fragments were found to be immunogenic in another preliminary study (196) that did not further evaluate diagnostic sensitivity and specificity.

Messick and Santos (85) identified several immunogenic *M. haemofelis* protein epitopes by screening whole-genome expression libraries and found five to be reactive to plasma derived from *M. haemofelis*-infected cats and not reactive to naïve cats, but further evaluation was not reported.

No studies on immunoreactive proteins of other haemoplasma species have been performed with the exception of *M. haemomuris* that had five major antigens identified on western blot (197). However, identification of the antigens, and potential interference of autoantibodies on the assay, could not be assessed as whole genome sequences were not available for *M. haemomuris* (197).

1.4 HOST SPECIFICITY, TRANSMISSION AND TRANSLATIONAL ASPECTS OF HAEMOPLASMAS

1.4.1 Host specificity of mycoplasmas

Mycoplasmas are generally host specific unless other factors, such as immunosuppression, allow them to cross wider species barriers (198). There is some evidence that genetically more heterogenous *Mycoplasma* species (e.g. *M. gallisepticum*) might be able to infect a wider host range (199). However, this evidence is based on identification of rather short bacterial DNA sequences from various hosts rather than actual inter-species transmission experiments with the same pathogen, and hence might reflect incomplete phylogenetic resolution of isolates (198).

1.4.2 Host specificity and transmission of haemoplasmas

The veterinary haemoplasmas also appear to be generally host specific (26). One study recently reported natural infection of *M. haemocanis* of a feline host, however this was based on 16S rRNA sequencing alone that cannot differentiate *M. haemocanis* from *M. haemofelis* (200).

A haemoplasma closely related to *M. suis* has been reported to infect farm workers and veterinarians in China, however published sequences of this isolate reflect a more heterogenous phylogenetic distribution than would be expected with one haemoplasma species (61). A haemoplasma closely related to *M. haemofelis* has been found in an immunocompromised human (60), as has an *M. ovis*-like haemoplasma (175). There is also some evidence to suggest that other haemoplasmas of non-human origin are possibly able to infect immunocompromised humans, as reported by Steer, Tasker (59). Yuan, Zhu (201) also found a *M. wenyonii*-like haemoplasma in pigs in China that were consistently negative on *M. suis*-targeting PCR assays. Fu, Shi (202) recently reported a 26.2% prevalence of '*Ca*. M. turicencis'-like haemoplasma in clinically healthy pigs.

However, very few strains of haemoplasmas have been fully characterised following isolation from their respective hosts. Given their wide distribution and underexplored genetic variability, interspecies transmission of haemoplasmas, or the presence of closely related and not fully

phylogenetically resolved haemoplasmas of species-specific pathogenicity, can equally not be ruled out (198).

To complicate matters, the natural transmission of haemoplasmas and the role of potential arthropod vectors remains unclear. Before PCR development, the louse *Polyplax serrata* was suggested as the natural vector for *E. coccoides* in mice as it survived up to 24 hours in fasted lice (203), but amplification within the louse host was then not demonstrated by PCR (204). The cat flea *Ctenocephalides felis* can contain '*Ca.* M. haemominutum' DNA (e.g. fleas collected from cats in the UK (205)) and Canadian fleas can contain *M. haemofelis* and '*Ca.* M. haemominutum' DNA (206). Transmission experiments using diluted blood samples containing '*Ca.* M. turicensis' inoculated subcutaneously into naïve cats to mimic flea-bites, showed infection in 4 out of 5 inoculated cats (207), suggesting that flea transmission could be possible. However, when fleas that were fed on *M. haemofelis* and '*Ca.* M. haemominutum'-infected cats then fed on 6 naïve cats in another study, transient *M. haemofelis* bacteraemia occurred in only one cat (208). Vectors have been further explored by Willi, Boretti (131) who found *M. haemofelis*, '*Ca.* M. haemominutum' and '*Ca.* M. turicensis' DNA in cat fleas, *Ixodes* spp. and *Rhipicephalus* spp. ticks collected from cats, but not in any unfed ticks collected from vegetation, suggesting that ticks may not act as biological vectors. Rodents were commonly positive for *E. coccoides,* but not feline haemoplasmas in the same study (131). Carriage of all three feline haemoplasmas was also reported in a small number of ticks collected from cats in the UK (209). No haemoplasmas were found in ticks collected from vegetation in Hungary (210) or apparently unfed ticks collected from dogs in the UK (151). There is however one report of '*Ca.* M. haemominutum' being detected in an unfed *Ixodes ovatus* tick in Japan (211).

In dogs, the tick *R. sanguineus* was assumed to be a natural vector of *M. haemocanis* (150). In a later study, using PCR, Tosa (Japanese fighting) dogs had a high prevalence of *M. haemocanis* with only 1 of 25 sampled male dogs being PCR-negative, compared to a prevalence of only 1.4% within the normal dog population in the same region (212), suggesting bite wounds as a possible route of transmission.

Social contact or inoculation with saliva/ingestion of haemoplasma-infected blood was not able to transmit *M. haemofelis* nor '*Ca.* M. haemominutum' infection in cats (207) although free-roaming lifestyle and male gender are reported risk factors for feline haemoplasma infection (98, 102, 106, 128, 146, 200).

Transplacental transmission has not been conclusively evaluated for cats or dogs, but blood contaminated semen may transmit porcine haemoplasmas (213). Blood transfusion transmission of haemoplasmas has been suggested by Gary, Richmond (214) for *M. haemofelis* and '*Ca.* M. haemominutum', but anticoagulated haemoplasma-infected blood was only able to establish lasting infection if inoculated after 24 hours at 4°C, whilst blood stored for one week only resulted in brief detection of '*Ca.* M. haemominutum' and no detection of *M. haemofelis* in the inoculated, naïve cats (214).

Few attempts have been made to assess host specificity using inter-host-species transmission experiments. Inoculation of *M. haemofelis*-infected feline blood did not establish infection in splenectomised mice, rats or dogs (122), nor in ruminants (215), but infection was based on blood-smear detection only. When inoculated with *M. haemocanis*-infected blood, cats did not show any signs of infection (anaemia or detectable organisms on blood smears) but these cats were able to infect naïve dogs through inoculation of their blood suggesting they were *M. haemocanis* carriers, but this study again only 'confirmed' infection by blood smear examination (126). More recently two healthy juvenile mice, two guinea pigs and one rabbit were inoculated with *M. haemofelis*-infected

blood and monitored by qPCR but no evidence of infection was found (216). One gerbil did amplify *M. haemofelis* 13 weeks after intraperitoneal inoculation, but bacteraemia was short-lived (Hicks, unpublished). Healthy laboratory mice did not amplify *M. suis* following intraperitoneal inoculation of *M. suis*-infected porcine blood (217).

1.5 RODENT MODELS IN INFECTIOUS DISEASE RESEARCH

1.5.1 Justification for animal models

With clinical haemoplasmosis being most relevant in cats and pigs, an alternative, smaller model of infection is highly desirable, especially in view of the unculturable status of haemoplasmas. In emerging diseases in humans, when there is a similar lack of data on pathogenesis and prevention of the disease, mouse models can provide vital insights into understanding disease mechanisms and aid the development of vaccines (218). Typically smaller animal models, such as murine models, are chosen based largely on their lesser cost and ethical burden and the ability to eliminate extrinsic and intrinsic factors within a study group by using inbred or even genetically modified strains (219). Ultimately, however, the aim would be for animal models to be replaced by non-animal methods, to refine them to use less invasive means and to reduce the number of animals required, as defined by the '3R principle' (220).

1.5.2 Definition of rodent model

In human medicine, animal or rodent models are "meant to emulate the biological phenomenon for a disease occurring in humans" (218). In veterinary research, without translational aspects, rodent models are less common but the same principles of extrapolation of data from one species to another may be applied.

When defining animal models, different criteria have been used to justify using research animals (218). Models should be homologous (i.e. share genetic similarities) in the response to the studied disease, and they should also resemble the clinical disease irrespective of its origin (referred to as analogy) and have high fidelity. Fidelity refers to how close the target species and the model are related and how much of the collected data (e.g. clinical outcomes after interventions) can be extrapolated (218). Homology and analogy generally describe the (animal) model species but may also be described for the pathogen when working with infectious agents.

In disease models involving highly adapted pathogens, using a naturally occurring pathogen, adapted to the model species (e.g. mouse) may require a higher degree of extrapolation as two different host species and two different species of pathogens are involved. However, depending on the research objective, using pathogens adapted to the model species may present a better model of the disease than infection of the model species with a less adapted 'unnatural' pathogen. For example, the feline immunodeficiency virus (FIV) model in cats is more suitable to assess HIV-related drugs as chimpanzees, despite being able to become infected with HIV do not develop disease like FIV-infected cats and HIV-infected humans (221). The use of naturally adapted pathogens in model species are called surrogate models and are defined by the substitution of an infectious agent by one that is more adapted to the host species in which it is to be investigated, and it may or may not be genetically related to the primary pathogen being investigated (222).

1.6 *IN VITRO* CULTIVATION OF THE HAEMOPLASMAS

1.6.1 Why cultivate haemoplasmas?

The absence of an *in vitro* cultivation system to generate more viable organisms is a major hindrance in haemoplasma research. Whilst development of a mouse model of haemoplasma infection may alleviate some of the ethical and financial challenges of only being able to grow haemoplasmas in their host animals, the mouse model would still not offer a feasible means of generating large-scale numbers of haemoplasmas for proteomics research or whole genome sequencing, as the size of laboratory mice precludes collection of large volumes of blood.

1.6.2 Known growth characteristics of related *Mycoplasma* species

Mycoplasmas are the smallest self-replicating free-living organisms and are currently thought to have developed from gram-positive bacteria of the class *Firmicutes* by reductive evolution (223). Due to their reduced small genomes, they lack certain metabolic capacities and *in vitro* cultivation has historically been very difficult. For instance, *Mycoplasma* spp. typically lack the ability to synthesise fatty acids or amino acids and require supplementation in the form of yeast extract or serum components (223). Prior to having whole genome sequences available, their nutritional requirements could only be determined by exclusion, leading to only one *Mycoplasma* species, *Mycoplasma laidlawii*, being cultivable in a defined medium that did not contain serum but that required the addition of nucleotides and fatty acids (224). However, even today, *in vitro* cultivation of many human pathogenic *Mycoplasma* spp. such as *Mycoplasma pneumoniae* (225) or *Mycoplasma genitalium* (226), require the addition of serum and other undefined extracts. As serum is a widely used additive for nutrient-rich cell culture media, mycoplasmas have gained importance as cell culture contaminants (227), and, due to their small size and tendency to form nanoforms in suboptimal growth environments (228), they typically cannot be removed by standard filtration from serum, which thus acts as source of mycoplasmas into culture media (229)

The genus *Phytoplasma* is also in the class *Mollicutes* but typically infects plants (230). A two-step *Phytoplasma* spp. *in vitro* cultivation system was recently reported but not described due to patent restrictions (231). A later publication by the same group compared their patented media compositions to commercially available media and confirmed growth in nutrient-rich *Mycoplasma* media, containing high salt concentration, horse serum, tryptone, soya peptone and incubation under microaerophilic conditions (127). Typically, *Mycoplasma* spp. grow in aerobic environments (232). The *in vitro* cultivation of spiroplasmas, another genus within the Mollicutes, faced similar problems although *Spiroplasma citri*, a plant pathogen, was able to grow in serum-free and yeast extract-free growth media (233). Most spiroplasmas require more complex media however, including insect cell lines in one case (234).

1.6.3 Previous attempts at cultivating haemoplasmas

Ever since their discovery in 1921 (235), researchers have tried to establish *in vitro* cultivation systems of haemoplasmas as an important means to further bacteriological research. Before PCR and molecular characterisation were available, various attempts to isolate *M. haemomuris* from rats for *in vitro* cultivation failed, as reviewed by Weinman (236). Ford and Eliot (237) described *M. haemomuris* as the causative pathogen of rat anaemia, but cultivation attempts in liquid, serum-containing media were inconclusive and likely comprised contaminants. The same research group later also failed to cultivate *E. coccoides* in identical media (238). Noguchi (239) reported isolating an organism from anaemic rats (*M. haemomuris)* in liquid 'leptospira medium' (containing rabbit serum, peptone and meat extract) that did not grow on other media available at the time. However, Noguchi failed to reproduce overt anaemia in rats inoculated with the subcultures. Marmorston-

Gottesman and Perla (240) obtained so-called 'growth' of *M. haemomuris* in Noguchi's 'leptospira medium' very rarely, but when they inoculated healthy rats with the cultured organism, they could only occasionally see organisms on blood smears. Additionally they were unable to demonstrate that their rats were free of *M. haemomuris* infection before inoculation. Seamer (9) reported the successful propagation (up to 16 consecutive passages) of *E. coccoides* in chicken embryos, which, when inoculated into mice, caused infection based on blood smear examination. However, Rikihisa, Kawahara (197) attempted to repeat the methodology of chicken embryo-inoculation of *M. haemomuris* and could not reproduce the results, despite PCR-based methods of detection being used. Hence, it is controversial whether the early reports of successful *in vitro* cultivation or propagation in chicken embryos truly represented organism survival and growth outside of the natural host.

Nonaka, Thacker (241) explored the *in vitro* cultivation of *M. suis* in various media and used different anticoagulants e.g. heparin and ethylenediaminetetraacetic acid (EDTA). They proposed an *in vitro* cultivation system based on serum-containing Eagle's medium and that EDTA caused *M. suis* to detach from erythrocytes and to lose its ability to infect. However, these findings were obtained by measuring glucose-consumption and examination of culture sediments by light microscopy only, which cannot be viewed as proof of successful *in vitro* propagation without further specific quantification of the cultured organisms. More recently Schreiner, Hoelzle (242) used an *in vitro* cultivation approach that was informed by genomic data being available for *M. suis*; they supplemented mycoplasma media (SP-4 and Hayflick's medium) with iron-carrier proteins, but could only establish maintenance of organism numbers and did not show viability of the 'maintained' organisms. In 2015, Hicks (216) attempted *in vitro* cultivation of *M. haemofelis* with hypoxanthine-supplemented specialist mycoplasma media described by Windsor and Windsor (243), but the initially observed growth over 4 days of culture could not be reproduced and viability of the cultured *M. haemofelis* was again not assessed.

1.7 OBJECTIVES OF STUDY

1.7.1 Rodent model of haemoplasma infection

A) To isolate and characterise rodent haemoplasmas from wild-caught animals in order to obtain viable inocula to infect laboratory rodents (Chapter 2).

B) To infect laboratory rodents with haemoplasmas sourced from wild-caught animals and monitor infection kinetics following naïve infection (Chapter 3).

C) To assess whether protection from re-infection with the same haemoplasma species or cross-protection occurs in the rodent model of haemoplasma infection (Chapter 3).

D) To establish a rodent model of haemoplasma infection and haemoplasmosis by characterising the rodent immune response to infection and pathological changes following infection (Chapter 4).

E) To identify the cytokine profile associated with protection from re-infection in the rodent model in order to guide further studies into haemoplasma immunology and vaccination (Chapter 4).

1.7.2 *In vitro* cultivation of haemoplasmas

F) To assess the viability of rodent haemoplasmas under *in vitro* conditions and identify *in vitro* conditions to support haemoplasma growth (Chapter 5).

2 ISOLATION AND IDENTIFICATION OF RODENT HAEMOPLASMAS AND INFECTION OF LABORATORY RODENTS

2.1 INTRODUCTION

2.1.1 Rationale for a murine model using rodent haemoplasmas

In the absence of an *in vitro* cultivation system (1.5), the establishment of a rodent model is highly desirable. The "3R principle" calls for researchers to replace animals, reduce their numbers and refine their experiments to produce valid data at minimal costs for the animals used (244). A rodent model of haemoplasma infection and haemoplasmosis would allow haemoplasma research with reduced ethical concerns of using research cats or other 'higher' animals, and the increased costs accompanying their use. An animal model using a less costly and more widely used laboratory animal model species would also allow the use of more laboratory research tools. Furthermore, as inbred strains are widely available in rodent species, studies using these strains as hosts can better limit individual variation when studying haemoplasma infection (1.4.3). This is a key requirement in defining complex immunological systems associated with infection and could help the development of vaccines and rapid serological assays. Having a relatively inexpensive source of viable haemoplasmas readily available would also benefit further research into *in vitro* cultivation and other *ex vivo* experiments.

With over 400 genetically stable inbred strains, typically raised under specified-pathogen-free (SPF) conditions, mice represent the most commonly employed animal model of disease worldwide (245). Furthermore, laboratory methods and reagents to study their immune system are relatively cheap and readily available. One inbred strain has been used in many disease models and as a wild type for genetically modified mice. This strain, C57BL/6, was established in 1921 after over twenty generations of inbreeding and has been maintained in various locations throughout the world (246). About 200 strains of rats are available. They are larger and usually more expensive than mice, but allow more serial samples to be taken, potentially reducing total animal numbers as experiments can be designed with each animal serving as its own control. This also allows the use of more genetically diverse (outbred) strains, which better reflect situations of naturally acquired infection. (246). Wistar rats (an outbred strain) represent the most commonly used strain of rat in medical research (247).

Surrogate models appear best suited to establish a rodent model of haemoplasma infection and/or haemoplasmosis as species-specific infection and pathogenicity must be considered in model selection (see 1.4). When replacing one naturally evolved host/pathogen combination with a physiologically and genetically related combination, data from the model might be extrapolated more readily than when using a non-adapted bacterial pathogen. However, identifying reasons for the lack of infection or pathogenicity of less adapted isolates (i.e. isolated from other species) is also of interest and may guide therapeutic or preventative reasoning.

2.1.2 Previous attempts to establish rodent models using non-rodent haemoplasma species

Attempts at creating rodent models using non-rodent haemoplasmas concentrated on using the most pathogenic haemoplasmas of cats and pigs, attempting to create a model using *M. haemofelis* and *M. suis* respectively. Reports from China suggest model establishment is possible using *M. suis* inoculation of Kunming mice. However, full methodologies are only described in the Chinese language and methods of monitoring infection appear to be semi-quantitative at best (248, 249). Furthermore, successful laboratory passages of *M. suis* into laboratory mice could not be repeated

by European research groups using quantitative methods of monitoring infection after inoculation of laboratory mice (216, 217). More specifically, *M. suis* was unable to establish infection in mice after inoculation of infected porcine blood but remained detectable for a few days following inoculation (217). Inoculation of fresh DMSO-preserved feline blood infected with *M. haemofelis* did not result in establishment of infection in juvenile mice, guinea pigs and rabbits (216).

2.1.3 Rodent haemoplasma infection

Rodent haemoplasma infection was first reported in 1921, as *Haemobartonella muris* in the blood of anaemic rats (235). The organism, also referred to as *Bartonella muris* at the time, could be transferred into normal laboratory rats through intraperitoneal inoculation of infected blood (237, 239). Infection rarely produced disease in otherwise healthy adult rats (236), but was able to induce anaemia in otherwise healthy, young (3-week old) rabbits, guinea pigs and mice through intraperitoneal inoculation of infected blood culture preparations (240). Experimental infection of rats was found to produce severe anaemia in combination with splenectomy, reaching mortality rates of up to 70 % (250).

Another eperythrocytic pathogen was found in 1928 in mice in Germany (2). The pathogen was referred to as *Eperythrozoon coccoides* and described as a "disease of white laboratory mice" and field mice that could be activated by splenectomy (2). In another later study, blood smear examination with Romanowsky-type stains revealed coccoid/ring-shaped bodies adherent to red cells of mice or free in the plasma, two to four days after splenectomy, but without overt clinical signs (238). The lack of clinical signs and the cytology (ring shape), that were later found to be artefacts (238), led to *E. coccoides* and *M. haemomuris* being considered separate species (236). Despite the ability of these two organisms to cross the species barrier between rats and mice (10, 240), *H. muris* was long considered a disease of rats whereas *E. coccoides* was considered a mouse pathogen (10, 194, 251, 252).

Eperythrozoon coccoides was considered a complicating factor in laboratory studies assessing the mouse immune response to non-haemoplasma infectious agents (252). Despite several studies on the immune response to *E. coccoides* infection, no surrogate model for veterinary haemoplasma infection was maintained (253-255).

Prior to the development of PCR in 1983 (256), specific and sensitive molecular diagnostic techniques were mostly unavailable, and haemoplasma experiments were based on observations using light microscopy, which is known to be unreliable (130, 257). In 1985, a Japanese research group (197) was able to passage *M. haemomuris* isolates from splenic suspensions of wild field mice (*Apodemus argenteus*) into ddY (outbred strain) laboratory mice, resulting in splenomegaly, inappetence and death. Isolates could be maintained *in vivo* by serial intraperitoneal injection of splenic cell suspensions, obtained 10 days post inoculation of the laboratory mice. Aliquots of these suspensions were then cryopreserved for future use. In 1997, these *M. haemomuris* (at the time also named *H. muris*) isolates were further passaged into Balb/C laboratory mice, producing similar outcomes of splenomegaly, anorexia and death seven to ten days post inoculation (197). Passage into Wistar rats was considered unsuccessful but PCR detected the presence of *M. haemomuris* DNA in their spleens seven to ten days post inoculation (197). However, another haemoplasma species, related but not identical to *M. haemomuris*, was found in a wild rat in another Japanese prefecture and was described as a novel species based on 16S rRNA gene sequencing (33). The same group went on to identify seven further isolates in rats and mice and proposed "subspecies ratti" and "subspecies musculi" within the species *M. haemomuris* (258). These subspecies currently hold 'Candidatus' status due to lack of comparative data available from other rodent haemoplasma

isolates (11). However, none of these rodent haemoplasma isolates was developed into a surrogate model of haemoplasma infection as it occurs in other veterinary relevant species.

2.1.4 Rationale for catching wild rodents

Our attempts to source cryopreserved, viable rodent haemoplasmas from research groups in Japan as discussed in 2.1.3, were unsuccessful. Haemoplasmas are considered fragile outside of their host (11, 197, 237) and infection of laboratory mice require otherwise innocuous samples for intraperitoneal inoculation without harming the animal. This minimises the use of blood or other possibly infected organ samples from collaborating groups as they could not be considered for attempts of rodent model establishment due to the lack of adequate sample collection, aseptic technique and cryopreservation (259).

Naturally occurring rodent haemoplasma infection has been described worldwide (34-36, 197, 258, 260) with haemoplasma infection rates as high as 21 out of 37 (57%) sampled mice (*Mus musculus*) reported (36). Despite no haemoplasma prevalence data of wild rodents being available from the UK, the findings by Hornok et al. suggest that rodent haemoplasmas are common and that sampling of wild rodents may provide a source of viable haemoplasmas for the attempted establishment of a surrogate model of haemoplasma infection or haemoplasmosis in veterinary relevant species.

2.1.5 Objectives

The objectives of this study were to capture wild *Muroidea* spp. (including *Mus musculus*, *Apodemus* spp. and *Microtus* spp.) in order to cryopreserve their blood in a way considered suitable for haemoplasma viability conservation. Generic haemoplasma PCR screening assays were used to detect rodent haemoplasma infection in sampled rodents that also had blood samples cryopreserved. Any positive samples were further characterised by sequencing and passaging into laboratory rodents (mice and rats) with the aim of establishing a surrogate model of haemoplasma infection and haemoplasmosis. Additionally, *M. suis* was evaluated for its species-specificity by trying to establish its infection in immunocompetent mice. Infection was monitored by specific, quantitative methods (generic haemoplasma qPCR, 1.2.5.2). These experiments were the first step to establish and validate a rodent model of haemoplasma infection with the aim to help further haemoplasma research into vaccination, serological detection and *in vitro* culture.

2.2 MATERIALS AND METHODS

2.2.1 Obtaining haemoplasmas through trapping of wild muroid species

2.2.1.1 Animals

Wild muroid species were identified by visual inspection upon removal from live traps. No species of any other classification than "least concern", as categorized by the International Union for Conservation of Nature (IUCN), was included in this study.

2.2.1.1.1 Ethical approval

All studies involving live trapping of muroid species were subject to ethical review (Animal Welfare Ethical Review Body of the University of Bristol) and approved under investigation licence UB/16/046. More specifically, this licence allowed wild capture and killing by cervical dislocation of the following species: *Mus musculus domesticus* (house mouse), *Apodemus sylvaticus, Apodemus flavicollis* (field mice) and *Microtus agrestis* (common vole). Our approved licensure was restricted to muroids being killed within 60 minutes of trap collection and a radius of up to 2 miles around the laboratory.

2.2.1.1.2 Trapping protocols for wild muroid species

Twelve Longworth small mammal live traps (NHBS Ltd, Totnes, UK) were loosely filled with hay and teaspoon-sized amounts of rolled oats, peanut butter and a piece of cucumber or apple (the latter having a high water content to provide a 'short-term water supply'). The radius in which the traps were placed included a veterinary teaching hospital, comprising small, large and farm animal facilities and various laboratory, storage and student housing facilities as well as hay barns and adjacent farmland. Buildings as well as open areas were included. Trap placement was adjusted weekly, based on trapping success and concurrent use of commercial rodent bait placements by others within the trapping area. Total trapping time was one month. Traps were monitored daily and inspected for captured wildlife as indicated by a closed front flap. Traps containing captured wildlife were returned to the laboratory within 60 minutes of collection and opened inside a clear polypropylene (PP) bag (Fisherbrand™, Clear Autoclave Bags, Thermo Fisher Scientific, UK) to avoid escape. Non-target species (i.e. molluscs, common shrews) and any target species showing obvious signs of late pregnancy or lactation were returned to the wild. Target muroid specimens were killed by cervical dislocation following fixation within a corner of the PP bag.

2.2.1.1.3 Blood sampling and cryopreservation

2.2.1.1.3.1 Blood samples for future inoculations

Immediately following cervical dislocation (2.2.1.1.2), muroids were removed from the PP bag and blood was collected through cardiac puncture with a 1ml heparinised syringe (Blood Gas Sampler, Westmed, USA, containing 25IU balanced heparin per syringe) equipped with a 5/8-inch 23G needle (Terumo®, Zhejang Kindly Medical Devices Ltd., PRC). Samples of approximately 100µl blood were mixed with 20% dimethyl sulfoxide (Fisher BioReagents™, Fisher scientific, UK) and frozen on dry ice. These samples were subsequently stored at -80°C for 31 to 52 days until their use in laboratory rodent inoculation (2.2.3.3.3 and 2.2.3.5).

2.2.1.1.3.2 Blood samples for haemoplasma screening qPCR

An additional sample of approximately 20µl of heparinised blood was obtained by expelling residual blood from the 1ml heparinised syringe (as described in 2.2.1.1.3.1) into a clean 1.5ml tube. Samples were frozen at -20°C until DNA extraction.

2.2.1.2 Screening for haemoplasma infection using a generic haemoplasma qPCR assay

2.2.1.2.1 DNA extraction

DNA was extracted manually, using silica membrane single-spin columns (NucleoSpin® Blood, Macherey-Nagel, UK). In brief, samples were defrosted and 10 µl of blood was diluted with phosphate-buffered saline (PBS) to a final volume of 200µl. Twenty-five µl of Proteinase K and 200µl of lysis buffer (BQ1) were added and the mixture incubated at 70°C for 20 minutes in a shaking incubator. Ethanol was added to a final volume of 410µl, mixed thoroughly and loaded onto a silica membrane column. The column was spun at 10,000 x g for one minute. The flow-through was discarded, 500µl of wash buffer 1 ('BW') was added and spun through the column at 10,000 x g for one minute. This was followed by an additional wash with 600µl wash buffer 2 ('B5') and spinning at 10,000 x g for two minutes to dry the silica membrane. Fifty µl of elution buffer ('BE') was brought to 70°C and loaded centrally onto the silica membrane and incubated for five minutes at room temperature (RT). After spinning at 10,000 x g for one minute, the column was discarded and the flow-through subject to generic qPCR and conventional PCR as outlined below. Samples not processed immediately were stored at -20°C until use.

2.2.1.2.2 Generic haemoplasma qPCR for rodent haemoplasmas

Two generic haemoplasma qPCR assays, as previously described (185), were used to screen for haemoplasma infection. The primers and probes for these assays had been designed to target a conserved region within several haemoplasma 16S rRNA genes with one assay amplifying the so-called 'haemofelis' (HF-) group of haemoplasma species (which contains *M. haemofelis*) and the other the so-called 'haemominutum' (HM-) group of haemoplasma species (which contains '*Ca.* M. haemominutum') (**Table 3**). However, cross-reactions of HF-group and HM-group species have been reported for these assays (the HF-group assay amplifying HM-group species more easily than vice versa) (185) and the HF-group assay was also found to amplify *Spiroplasma* spp. DNA in ticks (151). Primers and probes from a previously validated feline gene-specific PCR (261) were used as a host internal control to monitor for successful DNA extraction, confirm a lack of PCR inhibitors and that the qPCR set-up had been successful (**Table 3**). This assay had originally been designed to amplify the feline housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as its cDNA equivalent (261), and the targeted region was known to be identical in the mouse GAPDH gene, and at least similar in other rodents (216). Primers and probes were synthesised by Invitrogen, UK and the TaqMan probes were labelled with 6-carboxyfluorescein (FAM) at the 5' end and a Black Hole Quencher (BHQ)1 at the 3' end.

Assay	Forward Primer 5'-3'	Sense Taqman Probe 5'-3'	Reverse Primer 5'-3'
HF-group	GGAGCGGTGGAATGTGTAG	FAM-TYAAGAACACCAGAGGCGAAGGCG-BHQ1	GGGGTATCTAATCCCATTTGC
HM-group	GGGGCCAAGTCAAGTCATC	FAM-CCCGACGTTTGCACGATGTTACCAT-BHQ1	GCGAATTGCAGCCTTTTATC
Host internal control (feline/mouse GAPDH)	GCTGCCCAGAACATCATCC	FAM-TCACTGGCATGGCCTTCCGT-BHQ1	GTCAGATCCACGACGGACAC

Table 3 *Primers and probes (oligonucleotide) sequences used for quantitative detection of the so-called 'haemofelis' (HF)-group and the so-called 'haemominutum' (HM)-group and of host genomic DNA for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. HF, haemofelis; HM, haemominutum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxyfluorescein; BHQ, Black Hole Quencher.*

2.2.1.2.2.1 Quantitative PCR conditions

Each qPCR consisted of 2X GoTaq (Promega, Southampton, UK), 4.5mM $MgCl_2$, forward and reverse primers and probes for each of the three (HF-group, HM-group, GAPDH) assays (**Table 3**) with water to a final volume of 20µl. Probes were added to a final concentration of 100nM for the HF-group and HM-group assays and to a final concentration of 25nM for the GAPDH assay. Each primer was added to a final concentration of 200nM for the HF-group and HM-group assays, and 100nM final concentration for the GAPDH assay. Master mix reagents were aliquoted into 96-well PCR plates and 5µl of the eluted DNA sample was added to each well. As the same FAM fluorophore was used for both the generic haemoplasma HF-group and HM-group 16S rRNA gene and GAPDH gene qPCR assays, these reactions had to be run as parallel singleplexes, but were always maintained on the same qPCR plate. Positive control samples of known *M. haemofelis/*feline GAPDH gene copy numbers and negative control samples (water) were included on each plate. All qPCRs were performed on the same Mx3005P qPCR thermal cycling system (Agilent, UK) under the following conditions: Initial incubation for two mins at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. Fluorescence was detected at 520nm at the end of each annealing/extension phase.

2.2.1.2.2.2 Analysis of qPCR data

Analysis was carried out using the MxPro™ v4.10 software package (Stratagene, LaJolla, California, USA). Threshold fluorescence was set at 1000 relative fluorescence units (Rf) for both the HF-group and HM-group 16S rRNA gene qPCRs and the GAPDH gene qPCR. Data were imported into Excel (Microsoft® 2013) for subsequent analysis.

2.2.1.2.2.2.1 Normalisation of haemoplasma threshold cycle (Ct) values

Haemoplasma threshold cycle (Ct) values were normalised against host (GAPDH) Ct values to account for any variation in extraction efficiency, using the following formula:

$$\text{Normalised haemoplasma Ct values} = Ct - \Delta Ct^{H}$$

$$\text{where } \Delta Ct^{H} = Ct \text{ (host GAPDH)} - 20$$

The arbitrary Ct value of '20' was chosen as it was the closest integer to typical Ct values observed for DNA extraction from 10µl of blood and qPCR detection of host GAPDH (2.2.1.2). All haemoplasma Ct values were subsequently normalised to the host (GAPDH) Ct value of 20.

2.2.1.2.2.2 Calculation of relative haemoplasma genome copy numbers

Relative haemoplasma genome copy numbers were calculated using the formula:

$$\text{Relative haemoplasma genome copy number} = (1+E)^{\Delta Ct}$$

$$\text{where } \Delta CT = 40 - Ct \text{ and } E = \text{qPCR assay efficiency}$$

A PCR amplification follows an exponential curve that doubles the amount of product each cycle, assuming an efficiency of 1 (or 100%). The amplification can hence be expressed as 2^{Ct} or $(1+1)^{Ct}$. In relative quantification, a high Ct value (i.e. 40 cycles) is chosen to define the presence of a single copy of template DNA and the Ct differences to that value (ΔCT) are then used to calculate relative copy numbers. Assay efficiencies were 1.005 for the HF-group and 0.974 for the HM-group qPCR assays respectively (185).

2.2.2 Identification of rodent haemoplasma species

2.2.2.1 Amplification of additional rodent haemoplasma DNA

2.2.2.1.1 Origin of samples

The DNA samples used in the initial rodent haemoplasma species identification studies were derived from the original wild muroids following extraction from whole blood (2.2.1.2.1). For some of the subsequent amplification and sequencing reactions, DNA extracted from blood samples taken from laboratory animals during the first passages of cryopreserved wild rodent haemoplasma species was used instead.

2.2.2.1.2 Primers and PCR conditions to generate additional DNA sequence data

All primers were ordered from Metabion, Germany and reconstituted to 100µM prior to use.

2.2.2.1.2.1 Ribosomal gene fragment amplification and sequencing

2.2.2.1.2.1.1 Primers used for ribosomal gene fragment amplification

The 16S rRNA gene, the internally transcribed spacer region (ITS, intergenic spacer) and the 23S rRNA gene were targeted for amplification using generic *Mycoplasma* spp. primers and generic haemoplasma primers in various combinations. The primer sequences are given in **Table 4**. and their respective locations in relation to the ribosomal gene sequences is illustrated in **Figure 1**.

Primer name	Primer sequence 5'-3'
8-F[1]	AGAGTTTGATCCTGGCTCAG
1492-R[1]	GGTTACCTTGTTACGACTT
313-HBT-F[2]	ATACGGCCCATATTCCTACG
908-HBT-R[2]	TGCTCCACCACTTGTTCA
567-HF-group-F[3]	GGAGCGGTGGAATGTGTAG
680-HF-group-R[3]	CGTTTACCCTAATCTATGGGG
1061-HM-group-F[3]	GGGGCCAAGTCAAGTCATC
1199-HM-group-R[3]	CTATTTTCCGACGTTAAGCG
1309-F[4]	GTTCCCAGGTCTTGTACACA
2144-mod-R[4]	CAGTACTTGTTCACTATCGGTA
1446-R[5]	TACCTTGTTACGACTTAACT
1682-R[6]	TCGGCTCCATTTTCCAAGGC
2144-R[7]	CAGTACTTGTTCACTGGTA

Table 4 *Primer sequences used to amplify ribosomal 16S rRNA, internally transcribed spacer region and 23S rRNA gene fragments from rodent haemoplasma species obtained initially from wild muroids. The suffix -F indicates a forward primer and -R indicates a reverse primer. These primer sequences were derived from the publications identified by superscript numbers as follows: 1, Pitulle, Citron (262); 2, Criado-Fornelio, Martinez-Marcos (263); 3, Tasker, Peters (185); 4, Sasaoka, Suzuki (264); 5, Sashida, Sasaoka (33); 6, Harasawa, Hotzel (265); 7, Sashida, Sasaoka (258).*

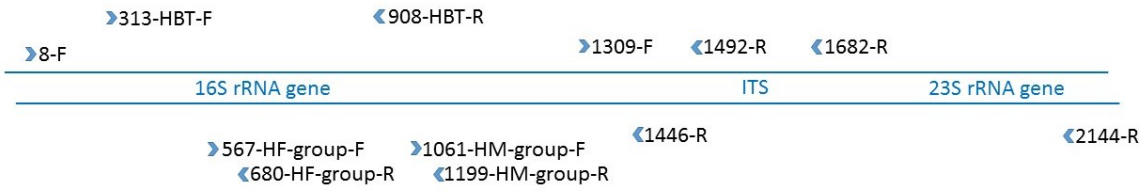


Figure 1 *Schematic diagram illustrating the location of the primer sequences used to amplify ribosomal 16S rRNA, internally transcribed spacer region and 23S rRNA gene fragments. The primer sequences and references are given in* ***Table 4****. ITS indicates the internally transcribed spacer region. The suffix-F indicates a forward primer and -R indicates a reverse primer and arrows indicate the direction of amplification.*

2.2.2.1.2.1.2 PCR conditions

Each PCR consisted of 2X GoTaq (Promega, UK), a final concentration of 4.5mM $MgCl_2$, 200nM each of forward and reverse primers with water to a volume of 20µl to which 5µl of template DNA was added. Reactions were carried out on a MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad, UK) under the following conditions: Initial incubation at 95°C for 2 minutes followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 50°C for 30 seconds and elongation at 72°C for 60 or 90 seconds followed by a final extension step of 72°C for 5 minutes. Negative controls (water) were included in each setup and positive controls (*M. haemofelis* or '*Ca.* M. haemominutum') were used with the respective assays (i.e. HF-group and HM-group respectively).

2.2.2.1.3 Separation and visualisation of DNA on agarose gels

2.2.2.1.3.1 Gel preparation

Agarose electrophoresis gels were prepared on the day of use or the day before and kept refrigerated at 4°C until use. Gels were prepared by mixing Tris Acetate-EDTA (TAE) buffer (Fluka Analytical, Sigma Aldrich) with 1 to 2% (w/v) agarose powder (Sigma-Aldrich) and the mixture was then brought to a boil. Upon cooling to approximately 45°C, 0.1µg/ml ethidium bromide (Bio-Rad Labs., UK) was added and after gentle mixing, the gels were poured as required.

2.2.2.1.3.2 Electrophoresis conditions

Samples of 5 µl PCR product were mixed with 1µl of loading buffer (DNA Gel Loading Dye (6X), Thermo Fisher, UK) immediately before loading onto the gel. EasyLadder 1™ (Bioline Ltd., London, UK) was used in duplicate on each gel to estimate amplicon size and confirm electrophoresis. Gels were submerged in TAE buffer and run at 50 to 80V for 30 to 90 minutes using a Horizon 11-14 or 58 gel electrophoresis system (Gibco BRL Life Technologies) with a Model 200/2.0 (Bio Rad Labs., UK) power supply unit.

2.2.2.1.3.3 Visualisation

Bands of DNA were visualised under ultraviolet light (BioDoc-It, Ultra-violet Products Ltd, UK) or blue light (Dark Reader™, Clare Chemical Research, USA) if downstream PCR applications (2.2.2.1.4) were planned.

2.2.2.1.4 Sampling for re-amplification PCR or nested PCR

When multiple bands were visualised following electrophoresis of PCR products, the following was performed in order to try and help further purify and re-amplify the product of choice for sequencing. The band closest in size to the expected size of the PCR product was 'stabbed' with a sterile needle that was then briefly submerged into a PCR master mix containing the same primers as previously or nested primers (using primers within the desired amplicon as based on **Figure 1**) to purify and/or amplify a more specific segment for sequencing.

2.2.2.1.4.1 Re-amplification PCR conditions

Whenever re-amplification was required, PCR conditions were identical to the original PCR (2.2.2.1.2.1.2) but reduced by 20 cycles whilst the annealing temperature was increased to 55°C to increase specificity.

2.2.2.2 Sequencing of rodent haemoplasma DNA

2.2.2.2.1 Purification of amplicons

Only strong, individual bands were considered suitable for sequencing reactions. Amplicons resulting from 2.2.2.1.2.1.2 and 2.2.2.1.4.1 were diluted 1:10 in nuclease-free water.

2.2.2.2.2 Sample submission for automated Sanger sequencing

Samples and primers were shipped at room temperature to DNA Sequencing and Services (College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) for automated Sanger sequencing, according to their recommended submission protocols.

2.2.2.2.2.1 Sequencing primers

The primers used for sequencing were the same as those used for PCR amplification of the gene segments (2.2.2.1.2). Sequence readouts were obtained from both ends of the amplicon to allow for a consensus sequence to be generated and help resolve any discrepancies.

2.2.2.3 Phylogeny of novel rodent haemoplasma sequences

2.2.2.3.1 Clean-up of sequence data

Electropherogram files were viewed using Bioedit v 7.2.5. (266) and inspected for any ambiguous readouts. Primers used to amplify and sequence the amplicon were aligned to the readout and removed from the resulting DNA sequence to account for stochastic elimination of mismatches on the respective primer-binding sites (as a few mismatches on the original template DNA could have been replaced by primer-generated amplicons during cycling reactions).

2.2.2.3.2 Comparison of DNA sequence data to online databases

Resulting consensus sequences were queried against the NCBInr nucleotide sequence database using the Basic Local Alignment Search Tool (BLAST) programmes (267) on the National Center for Biotechnology Information (NCBI) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.2.3.3 Construction of multiple sequence alignments

Multiple sequence alignments (MSA) of the 16S rRNA genes, ITS and 23S rRNA genes were generated using Mega7 (268) and the ClustalW algorithm (269), using the newly obtained DNA sequence readouts and previously published haemoplasma and *Mycoplasma* species sequences downloaded from Genbank (https://www.ncbi.nlm.nih.gov/genbank/). Accession numbers for these downloaded sequences are given within the results.

2.2.2.3.4 Construction of phylogenetic trees

Maximum-likelihood phylogenetic trees were constructed based on the Tamura-Nei model (270) using Mega7 (268) and treating nucleotide sequences as non-protein coding in inference of evolutionary history. Gaps were treated by complete deletion and phylogeny tested by the bootstrap method, set to 1000 replications.

2.2.2.3.5 Secondary structure prediction of RNA

Secondary structures were predicted using the Vienna RNA Websuite (271) using the method by Mathews and Zuker (272). Centroid representations were chosen over minimum free energy (MFE) predictions as they have been demonstrated to yield better accuracy in overall base pairing (273).

2.2.3 Infection of laboratory rodents with rodent haemoplasmas

2.2.3.1 Animals

2.2.3.1.1 Ethical approval

All studies involving infection of laboratory rodents were subject to ethical review (Animal Welfare Ethical Review Body of the University of Bristol) and covered by a Home Office project licence (PPL 30/2948).

2.2.3.1.2 Laboratory mice

Eight female, nine-week-old, SPF C57BL/6 mice were sourced from Harlan Laboratories UK Ltd., Loughborough, UK.

2.2.3.1.3 Laboratory rats

Three female, ten-week-old, SPF Wistar rats were sourced from Charles River Laboratories UK, Ltd., Margate, UK.

2.2.3.2 Housing conditions

2.2.3.2.1 Housing conditions for laboratory mice

Mice were housed in standard cages allowing for group sizes of up to four animals per cage. Cages were placed on an individually ventilated cage (IVC) unit and supplied with a standard laboratory diet (Lab Diet®, London, UK). Ambient temperature and humidity were monitored according to Home Office regulations (274). After haemoplasma inoculation, mice were monitored 2 to 3 times daily for any signs of ill health such as pallor, inappetence or ruffled fur.

2.2.3.2.2 Housing conditions for laboratory rats

Rats were housed in standard cages allowing for group sizes of up to three animals per cage. Rats were housed separately from other laboratory rodents and supplied with a standard laboratory diet (Lab Diet®, London, UK). Ambient temperature and humidity were monitored according to Home Office regulations (274). Upon entering the experiment (haemoplasma inoculation), rats were separated to eliminate possible haemoplasma transmission between experimental groups. Rats were monitored at least once a day for any signs of ill health such as pallor, inappetence or ruffled fur.

2.2.3.3 Acclimatisation, identification and screening for pre-existing haemoplasma infection in laboratory rodents

2.2.3.3.1 Acclimatisation and unique identification of laboratory rodents

Before entering any experiment, laboratory rodents were placed in the required groups and allowed an acclimatisation period of 4 to 7 days.

2.2.3.3.1.1 Identification of laboratory mice

Mice were uniquely identified by ear-notching procedures. In brief, mice had a 1mm punch removed from their outer ear pinnae, using a designated apparatus (Fisherbrand™ Animal Ear-Punch, Fisher scientific, UK) and were subsequently assigned a number within the cage: Mouse 1: left ear punched; mouse 2: right ear punched; mouse 3: no ear punch and mouse 4: both ears punched. This numbering system was maintained for all studies involving laboratory mice.

2.2.3.3.1.2 Identification of laboratory rats

Rats were uniquely identified by drawing circular markings on the proximal third of their tails (one circle = rat 1, two circles = rat 2, three circles = rat 3) which was renewed as necessary when markings began to fade.

2.2.3.3.2 Blood sampling procedures

2.2.3.3.2.1 Blood sampling in laboratory mice

Mice were habituated to blood sampling using a minimal restraint technique to allow serial blood sampling in the subsequent stages of the study. All mouse handling for blood sampling took place

within a fume extraction hood to avoid escape and contamination of the mouse facilities with infectious organisms.

All mice were habituated to various biscuits and cereal treats in the cage (**Figure 2, Panel A**). Mice were picked up from within the cages by allowing them to enter a cardboard tube and then raising the tube out of the cage, whenever possible, to avoid any potential additional stress, as recommended by current NC3R guidelines for mouse handling (275). Mice were also habituated to being positioned on a wire rack (as used to cover the top of the cage) with gentle touching of just the tail, whilst having access to treats that were just in front of the wire rack (see **Figure 2, Panel B**). The same treats were fed to all study mice on any given day. Daily blood sampling took place within a two-hour window (9am to 11am) for regular sampling intervals close to twenty-four hours. For the blood sampling procedure, mice were allowed to hold on to the wire rack and nibble on a treat placed in front of them, whilst gentle restraint was applied to the tail. The tail was then lifted and a tiny incision made over the lateral or the ventral tail vein using a scalpel blade (15 N/S, Swann Morton Ltd., Sheffield, UK) to allow a droplet of blood to form on the tail (**Figure 2**, **Panel C**). Gentle movement of the tail was used to encourage blood flow if the droplet did not appear quickly. The blood droplet was collected, by capillary action, into a heparinised (Haematocrit tubes, 80IU/ml Na-heparinised, Hawskley & Sons, Lancing, UK) microcapillary tube. Plain tubes were used for some studies. These microcapillary tubes were pre-labelled with a 10µl marking and upon filling of a tube with blood to that mark, the tube was placed into a clean 1.5ml tube containing 90µl of sterile PBS, and flushed with the PBS using a 1ml syringe (BD Plastipak™, Becton Dickinson S.A., Spain) and butterfly catheter tubing (Tro-Venoset, Troge Medical GmbH, Hamburg, Germany) so that the 100µl of blood/PBS mixture was all in the 1.5ml tube (**Figure 3**). The mice were then placed back into the cage and monitored for ongoing blood loss with the cage in a quiet location for approximately thirty minutes before placing the cage back onto the IVC rack if no bleeding or other signs of ill health were apparent.

Figure 2 *Minimal restraint technique for blood sampling in mice.*
*Habituation of mice to biscuit/cereal treats within the cage (**Panel A**) and on wire rack (**Panel B**);*
***Panel C**: Blood droplet formation on the ventral aspect of the tail.*

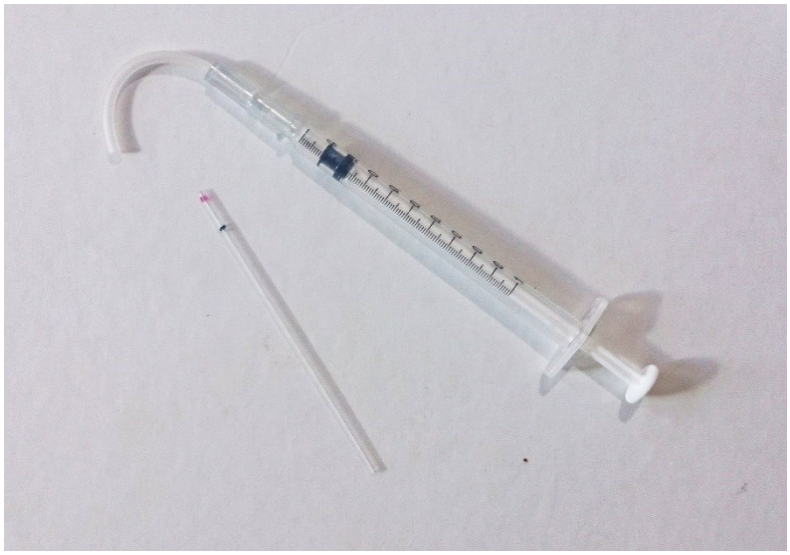


Figure 3 *Empty microcapillary tube and equipment used for flushing of blood from the microcapillary tubes. Eppendorf tubes were labelled and pre-filled with 90µl of PBS and opened prior to blood collection; the microcapillary tubes were filled with blood to a 10µl label mark and then placed into the Eppendorf tubes containing the PBS. A 1ml syringe was attached to a 23G butterfly catheter with the tube cut to leave approximately 2.5cm of tubing, as shown. The tubing was attached to the microcapillary tube and PBS flushed through by aspiration and expulsion so that the 100µl of mixed blood and PBS was expelled into the Eppendorf tube, ready for further analysis.*

2.2.3.3.2.2 Blood sampling in laboratory rats

Blood sampling in rats was carried out following a habituation phase (approximately one week) to allow the rats to become familiar with being handled by the experimenter. Rats were encouraged to allow human interaction by hand-feeding them biscuit treats within the cage and tolerate being picked up in return for further treats.

For the blood sampling procedure, rats were picked up and placed into a single use polypropylene bag (Transpack Ltd., Southhampton, UK), that had been cut and heat-sealed into conical shape with a 1cm hole cut at the tip to allow for air supply to the rat's face (**Figure 4, Panel A**). The cone was held closed at the base of the rat's tail and the rat was then placed on a soft surface (**Figure 4, Panels B and C**). A small stab incision was made with a 20G needle (Terumo) in the ventral tail vein, allowing a droplet of blood to form. Blood was then collected into a microcapillary tube as described for mice (see 2.2.3.3.2.1). This technique allowed for blood sampling from the rats with minimal restraint, avoiding compression of airways. Rats were subsequently released back into the cage, given a treat and monitored for ongoing blood loss for approximately thirty minutes.

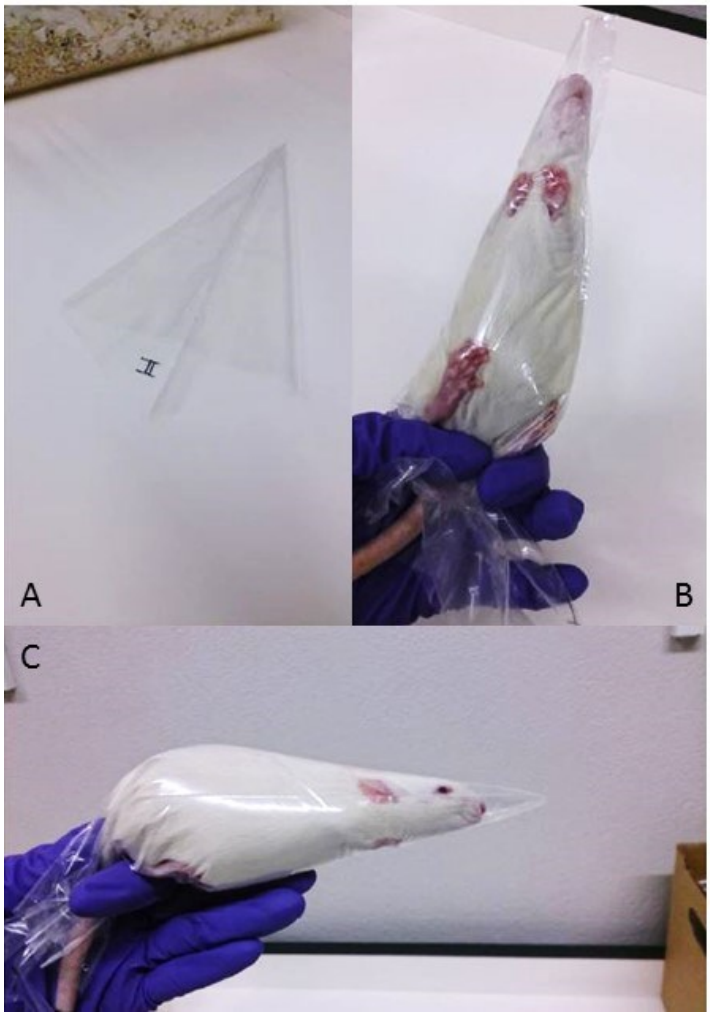


Figure 4 *One-person, minimal chest compression restraint technique for blood sampling in rats.*
Panel A: *Custom-made single use, conical polypropylene bag with the tip cut off to allow for enough air supply without compressing internal organs/airways during restraint.*
Panels B and C*: Restraint of rat inside the bag for blood sampling. Rats were subsequently placed onto a soft surface and blood sampled by making a small stab incision at the ventral aspect of the tail and allowing a blood droplet to form. Following blood sampling, rats were released from the bags and fed a biscuit treat.*

2.2.3.3.3 Detection of haemoplasmas in laboratory mouse blood samples pre-infection

In mice, the blood and PBS samples underwent DNA extraction and subsequent generic haemoplasma qPCR and GAPDH qPCR as described in 2.2.1.2. Analysis of qPCR data (2.2.1.2.2.2) was as previously described regarding normalisation and calculation of relative haemoplasma genome copy numbers.

2.2.3.3.4 Detection of haemoplasmas in laboratory rat blood samples pre-infection

In rats, blood and PBS samples underwent DNA extraction and generic haemoplasma qPCR detection and copy number calculation as described for mice (2.2.3.3.3). The host internal control GAPDH qPCR assay (2.2.1.2 and **Table 3**) was adjusted for sequence differences between the rat GAPDH gene sequence and the feline/mouse GAPDH gene (216). The modified reverse primer sequence is given below (**Table 5**). Primer synthesis was carried out by Invitrogen, ThermoFisher, UK.

Assay	Forward Primer 5'-3'	Sense Probe 5'-3'	Reverse Primer 5'-3'
Host internal control (rat GAPDH)	GCTGCCCAGAACATCATCC	FAM-TCACTGGCATGGCCTTCCGT-BHQ1	**GT**CAG**ATCCACAACG**GA**T**

Table 5 *Primers and probe (oligonucleotide) sequences used for quantitative detection of rat host genomic DNA for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Bases modified from the feline/mouse GAPDH qPCR assay (**Table 3**) are shown in bold and shaded text.*
GAPDH; glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxyfluorescein; BHQ, Black Hole Quencher.

2.2.3.4 Inoculation of laboratory mice with rodent haemoplasmas

Eight nine-week-old, SPF, female C57BL/6 mice were used for primary passaging of three novel rodent haemoplasma samples (each obtained from three different wild-caught rodents (2.2.1)) and for a porcine-derived *M. suis* sample (kindly donated by Prof. Hoelzle, Univerisität Hohenheim, Germany). Mice were split into four groups, each containing 2 mice, and each group was inoculated with either *M. suis* or one of three novel, rodent haemoplasma isolates and subsequently monitored for haemoplasma infection by qPCR.

2.2.3.4.1 Inoculation procedure in mice

Mice were inoculated intraperitoneally with the cryopreserved inoculum. Frozen inocula were allowed to defrost at room temperature for 5 to 10 minutes, diluted in PBS 1:2 and drawn up into a sterile 1ml syringe. Using a 5/8-inch 23G needle (Terumo), the inocula were inoculated into each mouse intraperitoneally. Mice were subsequently monitored for adverse reactions for thirty minutes.

2.2.3.4.2 Description of inocula used for inoculation of laboratory mice

Origin and composition of inocula used to inoculate the laboratory mice is given in **Table 6**.

Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	Inoculum dose in 0.1ml of inoculum per mouse	Number of mice inoculated
Mycoplasma suis	Blood collected from pig infected with *M. suis*	20% DMSO (v/v)	1:2	8.69E+10	2
Mycoplasma haemomuris	Blood from a wild-caught *Apodemus* spp.	20% DMSO (v/v)	1:2	1.43E+05	2
Eperythrozoon coccoides	Blood from a wild-caught *Apodemus* spp.	20% DMSO (v/v)	1:2	1.95E+05	2
Mycoplasma haemomuris-like haemoplasma	Blood from a wild-caught *Apodemus* spp.	20% DMSO (v/v)	1:2	3.79E+05	2

Table 6 *Origin and composition of inocula used to inoculate the laboratory mice. The M. suis inoculum was kindly donated by Prof. Hoelzle, Univerisität Hohenheim, Germany and shipped to the University of Bristol on dry ice and kept at -80°C until use. Inoculum doses were calculated as in 2.2.1.2.2.2. DMSO, dimethyl-sulfoxide.*

2.2.3.5 Inoculation of laboratory rats with rodent haemoplasmas

Three ten-week-old, SPF, female Wistar rats were used for passaging of three novel rodent haemoplasma isolates. As the original wild-caught rodent samples were inoculated into mice (**Table 6**), the rat experiment comprised a second passage of these novel rodent haemoplasma isolates, using samples derived from these mice. Each rat received one of the three haemoplasma isolates and was subsequently monitored for infection by qPCR.

2.2.3.5.1 Inoculation procedure in rats

Rats were inoculated intraperitoneally with cryopreserved inoculum. Frozen inocula were allowed to defrost at room temperature for 5 to 10 minutes and drawn up into a sterile 1 ml syringe. The inocula were inoculated into each rat intraperitoneally using a 5/8-inch 23G needle (Terumo). Rats were subsequently monitored for adverse reactions for thirty minutes.

2.2.3.5.2 Description of inocula used for inoculation of laboratory rats

Composition of the inocula used to inoculate the laboratory rats is given in **Table 7**.

Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	Inoculum dose (in 0.1ml) per rat	Number of rats inoculated
Mycoplasma haemomuris	Blood collected from C57BL/6 mouse (first passage)	20% DMSO (v/v)	-	3.26E+07	1
Eperythrozoon coccoides	Blood collected from C57BL/6 mouse (first passage)	20% DMSO (v/v)	-	5.77E+04	1
Mycoplasma haemomuris-like haemoplasma	Blood collected from C57BL/6 mouse (first passage)	20% DMSO (v/v)	-	5.27E+06	1

Table 7 *Inoculum composition and groups for primary rat infection experiment. Rats were housed together until the beginning of the experiment and housed separately, once inoculated. Inoculum doses were calculated as in 2.2.1.2.2.2. DMSO, dimethyl-sulfoxide.*

2.2.3.6 Monitoring of laboratory rodents for haemoplasma infection

2.2.3.6.1 Blood sampling regime in mice and rats (primary laboratory passages)

2.2.3.6.1.1 Monitoring via serial blood sampling for qPCR of mice

Serial blood sampling took place on days 0, 4, 7, 8, 11, 15, 17, 18, 24 and 35 post haemoplasma inoculation using the methodology described earlier (2.2.3.3.2.1) with heparinised microcapillary tubes. The mixture of heparinised blood and PBS was stored at 4°C for up to 24 hours until further processing.

2.2.3.6.1.2 Monitoring via serial blood sampling for qPCR of rats

Serial blood sampling procedures took place on days 0, 2, 4, 6, 8, 10, 11, 12, 14, 16, 17, 18, 21, 22, 23 and 24 post haemoplasma inoculation, using the methodology described above (2.2.3.3.2.2) with heparinised microcapillary tubes. The mixture of heparinised blood and PBS was stored at 4°C for up to 24 hours until further processing.

2.2.3.6.2 Determination of relative rodent haemoplasma blood copy numbers in mice and rats

The method used for DNA extraction (2.2.1.2.1) from the blood samples, and the qPCRs used for haemoplasma screening in mice (2.2.3.3.3) and rats (2.2.3.3.4) were as described previously. DNA samples from mice, inoculated with blood containing *M. suis* were analysed using the HM-group generic assay and all other mice and rats were analysed using the HF-group generic assay as dictated by phylogeny and previous detection of the novel isolates (2.3.2.3). Threshold fluorescence and data

generation, normalisation of relative genome copy numbers and calculation of relative copy numbers, using normalised Ct values, was carried out as described above (2.2.3.3.3).

2.2.3.7 Collection of inocula from rodents for use in further studies

Mice were killed by exsanguination under terminal anaesthesia at perceived timepoints of high haemoplasma parasitaemia to collect inocula for future studies. Mice inoculated with *M. haemomuris* were killed on day 8 post infection and mice inoculated with *E. coccoides* were killed on days 18 and 35 post infection. No inocula were sourced from the mice inoculated with *M. suis*, as long-standing infection was not successful, and these mice were subsequently rehomed in accordance with regulatory procedures (2.2.3.1.1).

The protocol for exsanguination comprised putting the mouse into an induction chamber and exposing it to halothane (Sigma-Aldrich Company Ltd., Gillingham, UK) in oxygen at 3% (v/v). Upon induction of general anaesthesia, as indicated by a lack of pain perception and spinal reflexes as assessed by withdrawal and tail-flick responses, the mouse was placed in dorsal recumbency and a mask connected to the anaesthetic machine was placed over the mouse's mouth and nose. Halothane concentration was subsequently reduced to 2% (v/v) or maintained as required for general anaesthesia. Using a 1ml heparinised syringe (Blood Gas Sampler, Westmed, USA, containing 25IU balanced heparin per syringe) and a 5/8-inch 23G needle (Terumo) mice were exsanguinated by cardiac puncture by inserting the needle at a 30° angle aiming craniodorsally underneath the xiphoid. Care was taken to avoid multiple needle sticks to reduce contamination and haemolysis. Post-mortem examinations were carried out on all killed mice. Splenomegaly was defined as a splenic weight over 0.4% bodyweight (276).

Rats had aliquots of 100µl of heparinised blood taken for cryopreservation at various days during the experiment as described above (2.2.3.3.2.2) and were subsequently rehomed in accordance with regulatory procedures (2.2.3.1.1) as no long-standing infection was established in rats.

Collected blood samples from rats and mice were mixed with 20% DMSO (v/v) and frozen on dry ice before transferring them to a freezer at -80°C as described for blood samples from wild-caught muroids above (2.2.1.1.3.1).

2.3 RESULTS

2.3.1 Trapping and sampling

Twenty-one wild muroids were trapped during the observation period. Six were identified as *Microtus* spp. (i.e. voles) and 15 as *Apodemus* spp. (i.e. field mice). Five of the 15 blood samples obtained from the *Apodemus* spp. yielded positive results on the HF-group qPCR assay. Three of these five samples also had (weak) positive results on the HM-group qPCR assay (data not shown) , which was attributed to cross-reaction of the qPCR assay as has been previously reported (185). Sample origin, calculated normalised relative haemoplasma copy numbers and eventual sample identification (see 2.3.2) are given in **Table 8**.

Sample origin	Normalised relative 16S rRNA gene copies per µl blood	Sample identification
Apodemus spp. #6	2.86E+03	*Mycoplasma haemomuris* (100% identity/coverage, E value 3E-29, AB820289.1)
Apodemus spp. #8	2.86E+04	*Mycoplasma haemomuris* (100% identity/coverage, E value 3E-29, AB820289.1)
Apodemus spp. #9	3.43E+03	Putative rodent haemoplasma (*Mastomys erythroleucus* isolate, 90% identity, 97% coverage, E value 2E-16, KU697344.1)
Apodemus spp. #13	7.57E+03	Putative rodent haemoplasma (*Mastomys erythroleucus* isolate, 100% identity/coverage, E value 3E-29, KU697344.1)
Apodemus spp. #18	3.91E+03	*Eperythrozoon coccoides* (100% identity/coverage, E value: 6E-37, AY171918.1)

Table 8 *Description of haemoplasma isolates from wild-caught Apodemus spp. (field mice). Normalised relative genome copy numbers/µl blood were based on HF-group assay and calculated as described earlier (see 2.2.1.2.2.2.2). Sample identification was based on the following: the primer sequences were removed from the original 113 base pair PCR amplicon derived from the positive generic HF-group haemoplasma leaving a 74 base pair amplicon to query against the BLASTnr database for identification. As indicated, all amplicon sequences except for isolate #9 were covered 100% within the database which means identity was calculated over the entire sequence.*

2.3.2 Identification and phylogeny of novel rodent haemoplasma isolates

2.3.2.1 Initial identification following qPCR screening

Samples positive on the HF-group qPCR assay were initially identified based on data obtained following sequencing of the 16S rRNA gene 113 base pair amplicon derived from the qPCR assay, and results are shown in **Table 8**. The 74 base pair sequences (amplicon without primers) used for BLASTn queries are given in **Appendix B, Table A-B1**.

Two of the samples (#6 and #8) were identified as *M. haemomuris*, one (#18) as *E. coccoides* and two (#9 and #13) most resembled rodent haemoplasma isolates described from multimammate mice (*Mastomys erythroleycus*) and other unidentified rodents in Senegal, Brazil and other locations worldwide. Sample #13 was also found to be 99% identical with a previously unpublished "vole haemoplasma species", isolated from wild-caught voles in the UK (277).

Further sequence data were required as sample #9 also had 88% identity to *M. haemofelis/M. haemocanis* species (Genbank accession numbers MH447082.1 and MG594502.1 respectively) and sample #18 had 100% identity to '*Ca.* Mycoplasma turicensis' (accession number Y046312.1), reflecting the high sequence conservation of the assay's target region that was below the variation required for species-resolution.

2.3.2.2 Derivation of further ribosomal gene sequences

Following initial identification based on the short HF-group qPCR product sequences, all of the novel rodent haemoplasma isolates (**Table 8**) had longer 16S rRNA gene sequences of approximately 600 base pair length amplified, using primers 313-HBT-F and 908-HBT-R (263). This was required due to

the small size of the previously used amplicon (**Table 8**) that did not reliably identify isolates at species level. Upon BLASTn queries of the cleaned sequence readouts, isolates #6 and #8 were found to have 99% identity to *M. haemomuris* (AB820289.1 and others). Isolate #9 had 94% identity with a haemoplasma species (KT215638.1) isolated from the spleens of Brazilian wild rodents (68) and 92% identity with *M. haemomuris* followed by '*Ca.* Mycoplasma haemomacaque' at 91% identity. Isolate #13 had 99% identity with another sample (KT215630.1) from the same Brazilian biome sampled by Gonçalves et al. 2015. Isolate #13 had 96% identity with *M. haemomuris*, followed by '*Ca.* Mycoplasma haemohominis' with 95% identity. It also had 99% identity with the previously mentioned unpublished "vole haemoplasma" isolate and 98% 16S rRNA gene identity with a gerbil haemoplasma isolate in Israel (278). Isolate #13 was subsequently termed "*M. haemomuris*-like haemoplasma" relating to its closest phylogenetical neighbour. Isolate #18 had 99% identity with *E. coccoides* (AY171918.1).

Three of these novel rodent haemoplasma isolates (#6, #13 and #18) were chosen for passaging into laboratory mice (2.2.3) and further sequencing work. The resulting DNA sequences for the partial 16S, the ITS region and the partial 23S rRNA genes of these isolates comprised 2041 base pairs for *M. haemomuris* #6, 1626 base pairs for *M. haemomuris*-like #13 and 1327 base pairs for *E. coccoides* #18 and are given in Appendix B. Based on the near complete 16S rRNA gene sequences, species identity was 98% to the Brazilian rodent haemoplasma (KT215638.1 and others) for isolate #13. Considering increased coverage, this isolate was now 97% identical to the proposed subspecies 'ratti' and 95% identical to the proposed subspecies 'musculi' of *M. haemomuris.* The *E. coccoides* isolate #18 was 97% identical to other *E. coccoides* isolates and *M. haemomuris* isolate #6 was 99% identical to the proposed subspecies 'ratti' and 'musculi' of the same genus. Species identification based on ITS or 23S rRNA gene sequences was unsuccessful due to the lack of sequence data available from other studies for comparison.

2.3.2.3 16S rRNA gene phylogeny of the novel rodent haemoplasma isolates

Phylogenetic relationships between the three novel rodent haemoplasma isolates obtained in this study for further passaging, and previously published haemoplasmas based on near complete 16S rRNA gene sequences are illustrated in **Figure 5**. A phylogenetic tree, constructed using a 464 base pair sequence within a variable region of the 16S rRNA gene that was available for all five novel- and several previously isolated rodent haemoplasmas did not reliably resolve established species such as *M. haemofelis* versus '*Ca.* M. haemobos' or '*Ca.* M. turicensis' versus *E. coccoides* (Appendix B, **Figure A-B1**).

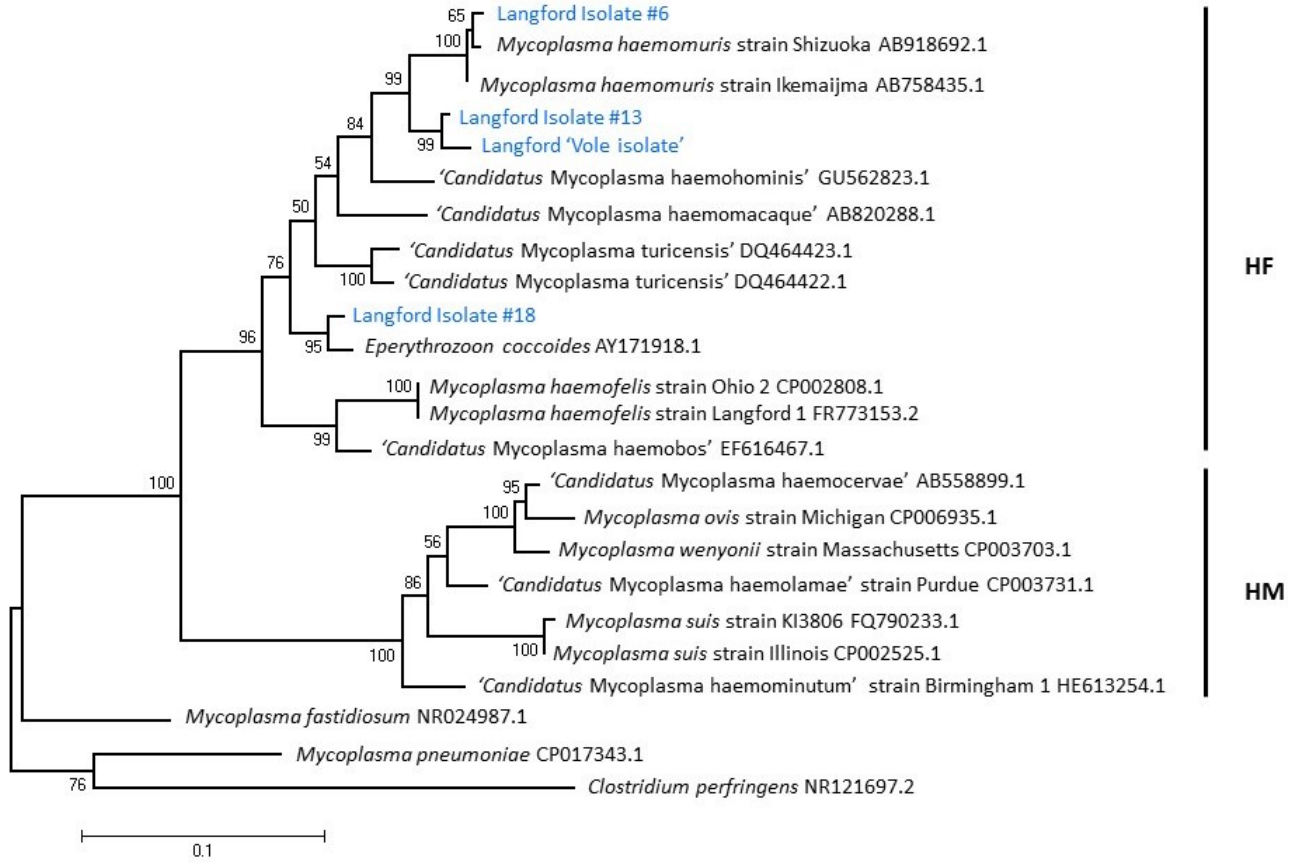


Figure 5 *Phylogenetic tree of near complete/complete 16S rRNA gene sequences of novel and previously published haemoplasma sequences, rooted by other Mycoplasma spp. and Clostridium perfringens. Evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model (270) and conducted in Mega7 (268). The dataset was sampled 1000 times and bootstrap values are given as percentages where nodes occur. The scale bar indicates evolutionary distance. Accession numbers are given next to species names. The novel rodent haemoplasma isolates derived from the current study (Langford #6, #13 and #18 as well as a previously unpublished 'vole haemoplasma' isolate (277)) are highlighted in blue. HF, haemofelis group of haemoplasmas; HM, haemominutum group of haemoplasmas*

Based on this 16S rRNA gene sequence phylogeny, isolate #13 formed a "vole cluster" independently to other *M. haemomuris* isolates, and isolate #18 was confirmed as mostly likely being *E. coccoides*. Isolate #6 clustered with two *M. haemomuris* isolates included in the analysis but appeared closer to the Shizuoka strain. The Shizuoka strain was originally isolated from field mice and was proposed as a subspecies within *M. haemomuris* based on sequence data from the ITS region and rnpB genes (279, 280).

2.3.2.4 Internallly transcribed spacer region phylogeny of M. haemomuris isolates

The highly variable ITS region between the 16S and 23S rRNA genes was targeted to closer determine the relationships of the novel *M. haemomuris* isolate to other rodent haemoplasma isolates. As amplification of ITS failed for *E. coccoides* #18, only ITS sequence data from isolates #13 and #6 were included. Sequence alignment of the ITS region (equivalent to positions 548178 to 548403 of *M. haemofelis* accession number FR773153.2) of our novel rodent haemoplasmas and other haemoplasmas from within the HF-group is given in **Figure 6**.

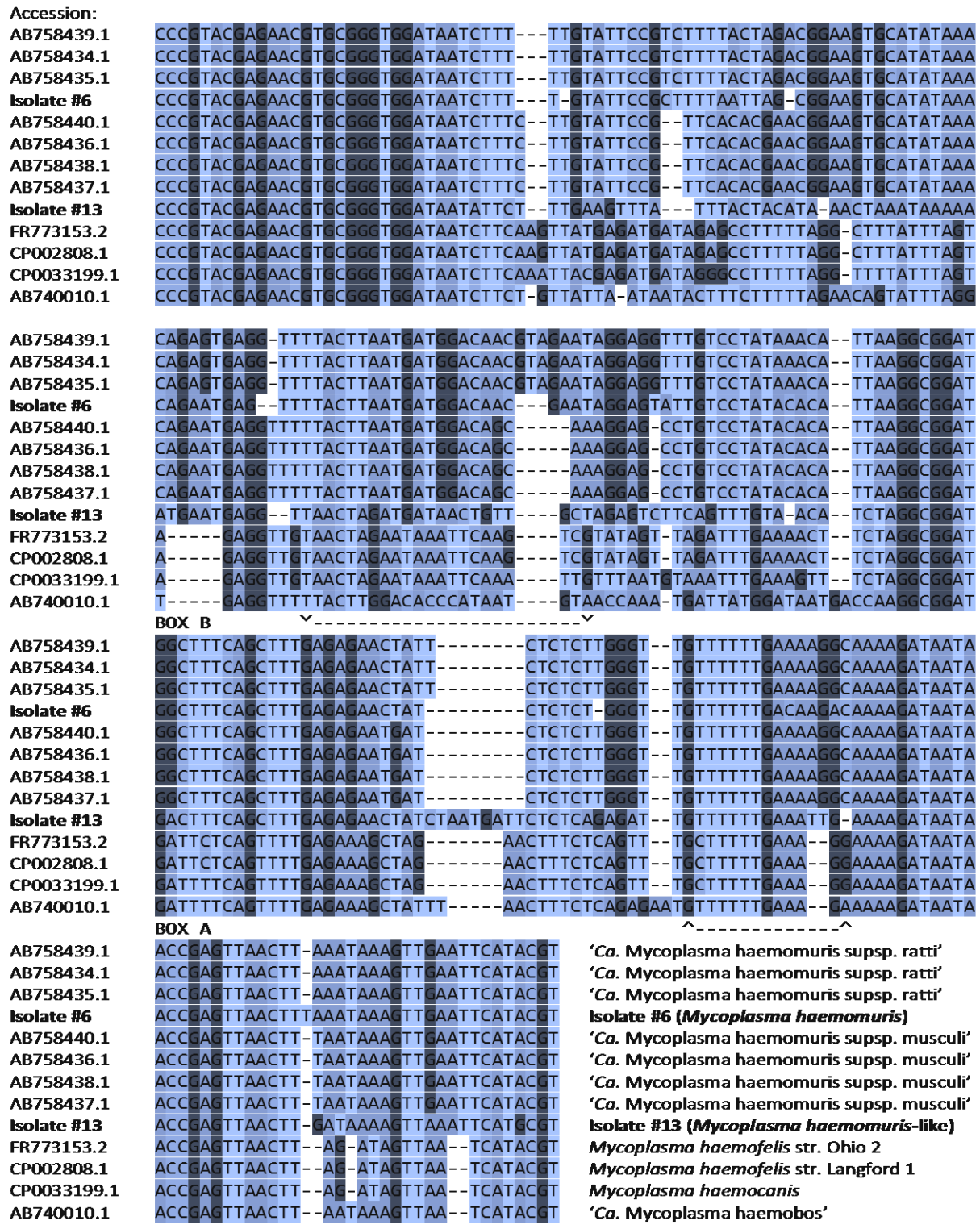


Figure 6 *Nucleotide sequence alignment of the internally transcribed spacer (ITS) sequences of the novel rodent haemoplasma isolates (#6, #13) and previously published haemoplasma species within the HF-group of haemoplasmas. Sequences were aligned using Clustal W in Mega7 (268). Dashes indicate gaps between adjacent nucleotides caused by the alignment. Sequences are equivalent to positions 548178 to 548403 on M. haemofelis accession number FR773153.2. The 'Box A' and 'Box B' internal promotor motifs (281) are indicated with arrows/dashes within the alignment. Box A was present in all isolates but Box B missing in isolate 13. Ca., Candidatus.*

The novel rodent haemoplasma isolate #6 appeared to reside within the *M. haemomuris*-group of isolates, with 93 % identity to the '*Ca.* Mycoplasma haemomuris subspecies ratti' group of isolates and 90 % identity to the '*Ca.* Mycoplasma haemomuris subspecies musculi' group (as determined via

BLASTn queries). Other closely related species listed in **Figure 6** demonstrated similar levels of sequence conservation (i.e. *M. haemofelis* and *M. haemocanis*; both 94% identity). The novel rodent haemoplasma isolate #13 demonstrated higher variability, with the conserved 'Box A'-like and 'Box B'-like regions (281, 282) conserved for isolate #6 but disrupted by additional nucleotides for isolate #13. Hypothetical secondary structures of the ITS regions for the novel isolates are shown in **Figure 7** and **Figure 8** and hypothetical structure predictions of the conserved 'Box A'-like and 'Box B'-like regions within the ITS are given in **Figure 9**.

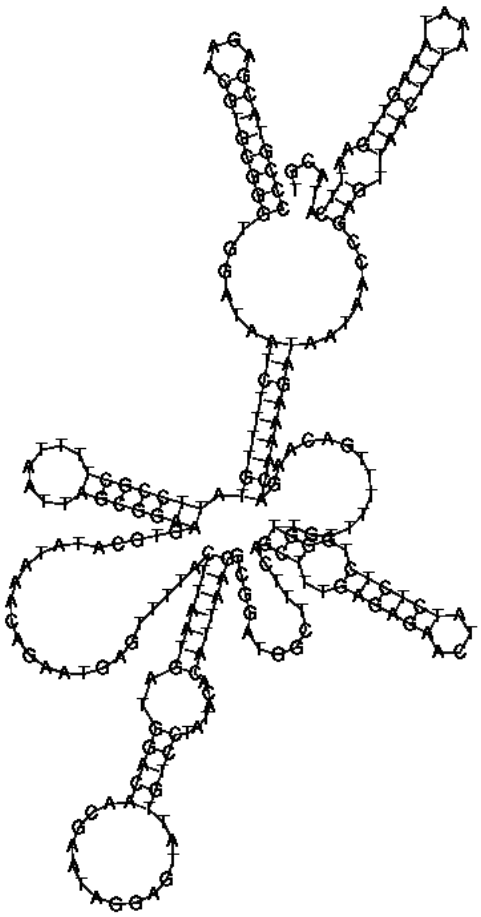


Figure 7 *Secondary structure prediction of the internally transcribed spacer (ITS) region of the novel M. haemomuris isolate #6. Centroid representations were calculated, using the Vienna RNA Websuite (Lorenz et al. 2011) after the method by Mathews and Zuker (Mathews et al. 2004). Centroid representations were chosen over minimum free energy (MFE) predictions as they have been demonstrated to yield better accuracy in overall base pairing (Rogers and Heitsch 2016).*

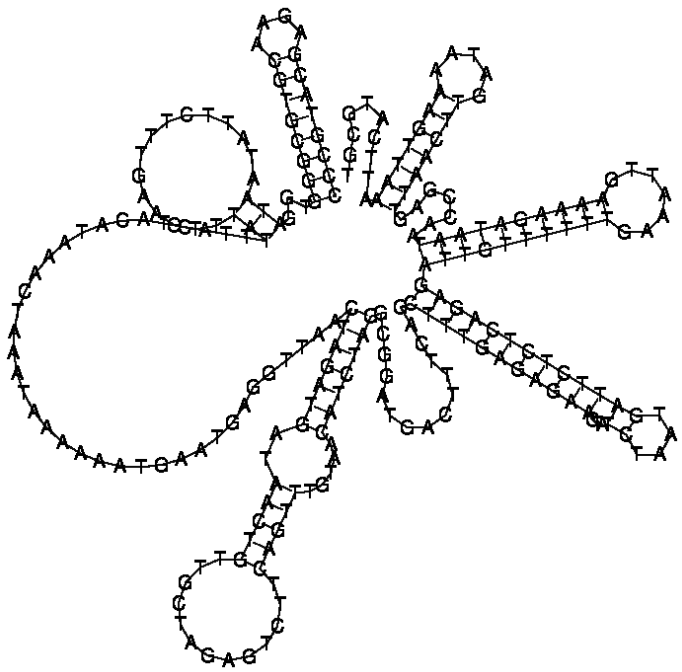


Figure 8 *Secondary structure prediction of the internally transcribed spacer (ITS) region of M. haemomuris-like haemoplasma isolate #13. Centroid representations were calculated, using the Vienna RNA Websuite (Lorenz et al. 2011) after the method by Mathews and Zuker (Mathews et al. 2004). Centroid representations were chosen over minimum free energy (MFE) predictions as they have been demonstrated to yield better accuracy in overall base pairing (Rogers and Heitsch 2016).*

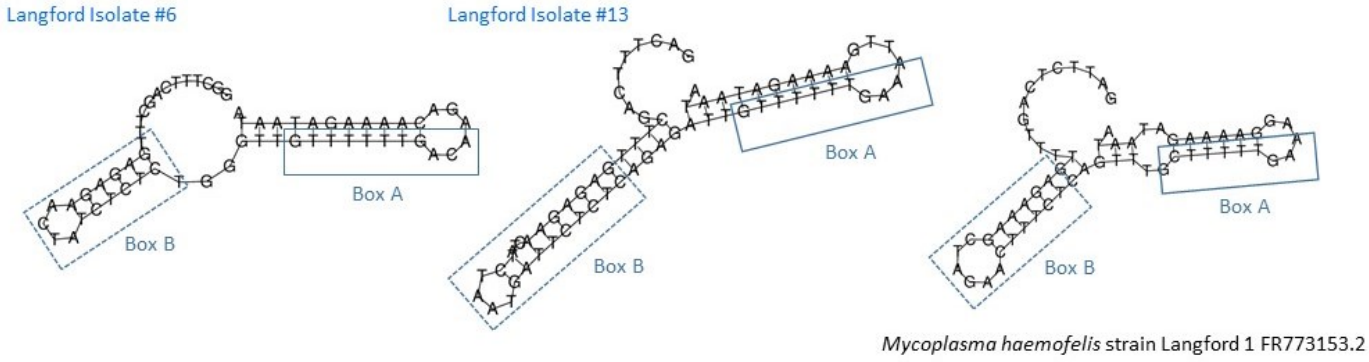


Figure 9 *Hypothetical secondary structure of 'Box A'-like and 'Box B'-like sequences (281, 282) within the internally transcribed spacer (ITS) region of the two novel rodent haemoplasma isolates Langford Isolate #6 and #13. M. haemofelis accession number FR773153.2 is shown for comparison. Centroid representations were calculated, using the Vienna RNA Websuite (Lorenz et al. 2011) after the method by Mathews and Zuker (Mathews et al. 2004). Isolate #13 had a less conserved primary sequence but secondary structure still revealed two conserved loops that could serve as internal promotors and are conserved among microorganisms.*

Phylogenetic relationships within the HF-group of haemoplasmas, based on the highly variable ITS region and concentrating on previously published *M. haemomuris*-isolates, are given in **Figure 10**.

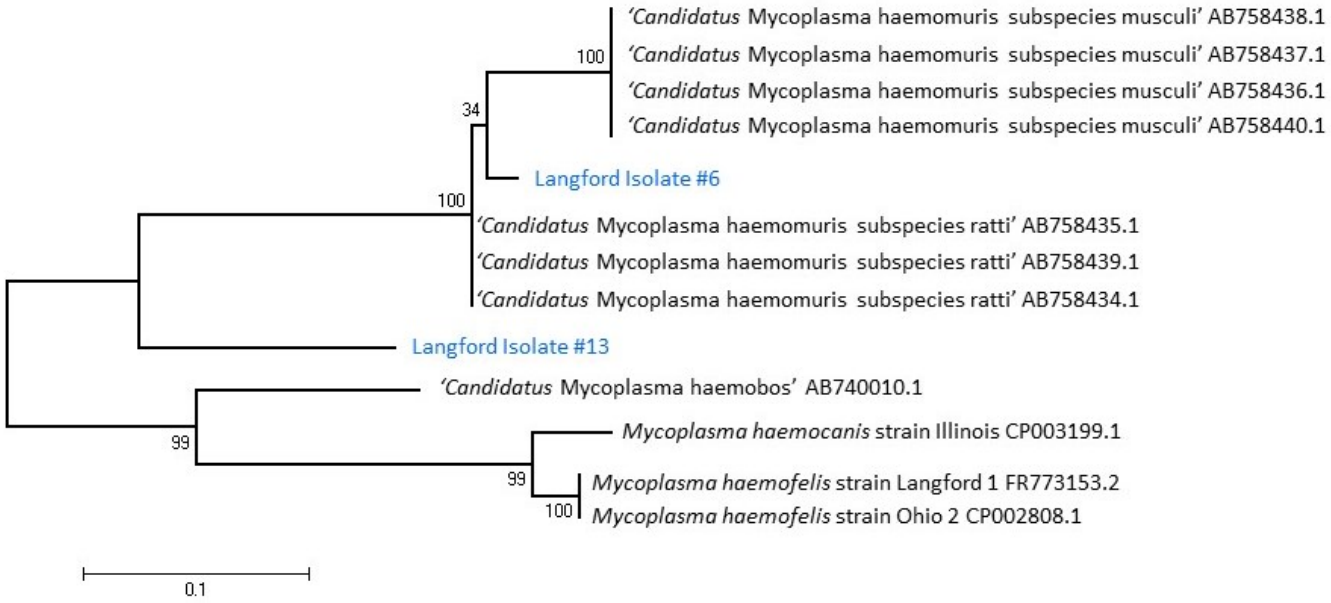


Figure 10 *Phylogenetic tree of internally transcribed spacer (ITS) sequences from rodent haemoplasma isolates, rooted by other haemoplasmas from within the HF-group. Evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model (270) and conducted in Mega7 (268). The dataset was sampled 1000 times and bootstrap values are given as percentages where nodes occur. Nodes with support values of less than 50 were left in place for illustrative reasons. The scale bar indicates evolutionary distance. Accession numbers are given next to species names. Isolates derived from this study are highlighted in blue.*

Isolate #6 barely split from the '*Ca.* M. haemomuris subspecies ratti' group of isolates (branch support of 34%) but was well differentiated from the isolates, previously identified from Japanese field mice. Isolate #13 remained an isolated species within the HF-group haemoplasmas when assessing the ITS region.

2.3.3 Haemoplasma passaging into laboratory rodents

2.3.3.1 Initial passage of novel rodent haemoplasmas into laboratory mice

2.3.3.1.1 Clinical signs and post-mortem examinations

No clinical signs of ill health were noted in any mice throughout the experiment.

Both mice, successfully infected with *M. haemomuris* (isolate #6), showed splenomegaly (defined as splenic weight above 0.4% bodyweight) at the time of exsanguination at eight days post inoculation. Splenic weights were 1.6 and 1.5% bodyweight. One of the two mice, having successfully yielded amplication of a novel *M. haemomuris*-like haemoplasma (isolate #13), had splenomegaly (splenic weight of 1.6% bodyweight) at the time of killing at eight days post inoculation and in the absence of other significant post-mortem findings. The second *M. haemomuris*-like haemoplasma-infected mouse had no abnormal findings on post-mortem examination on the same day (splenic weight comprising 0.6% bodyweight). One of the two mice inoculated with *E. coccoides* (isolate #18) had no abnormal post-mortem findings at 18 days post inoculation and no data ere available for the second *E. coccoides*-inoculated mouse as it was rehomed after the experiment following repeatedly negative qPCR results.

2.3.3.1.2 Haemoplasma genome copies in mouse blood samples

Normalised relative haemoplasma (*M. haemomuris*, *M. haemomuris*-like haemoplasma, *E. coccoides* and *M. suis*) genome copies per µl blood after inoculation of naïve C57BL/6 mice are shown in **Figure 11**.

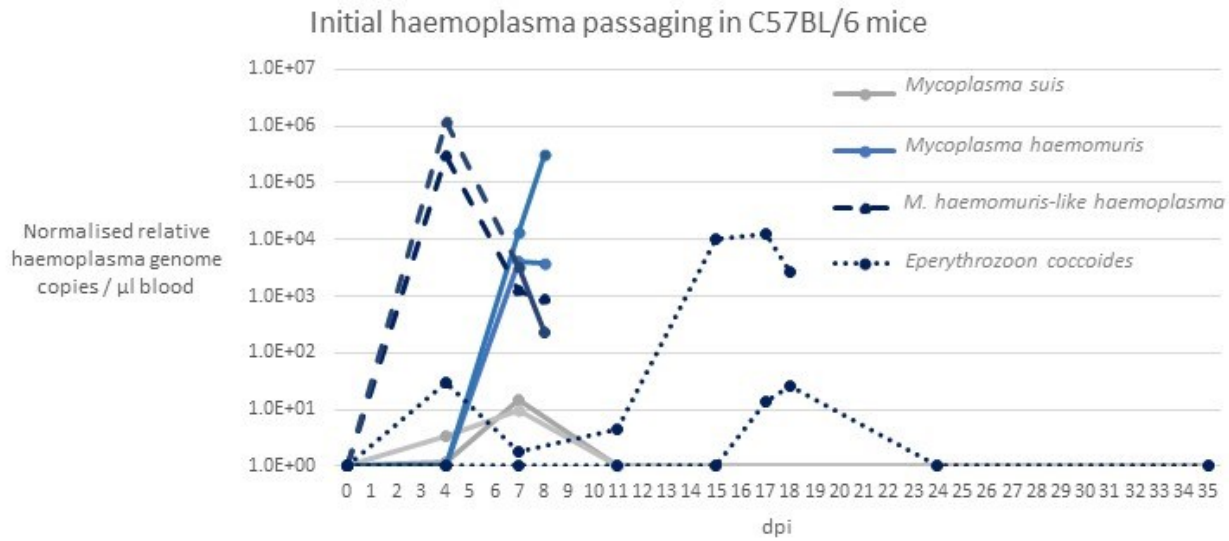


Figure 11 *Haemoplasma genome copies during initial passaging into C57BL/6 mice. Days post inoculation are plotted on the x-axis and normalised relative haemoplasma genome copies per µl blood are plotted on the y-axis. Connected dots represent individual mice. Solid grey lines: M. suis; solid light blue lines: M. haemomuris; dashed dark blue lines: M. haemomuris-like haemoplasma; dotted dark blue lines: E. coccoides; Mice inoculated with M. haemomuris and M. haemomuris-like haemoplasma were killed on day 8 of the experiment and one mouse, inoculating with E. coccoides was killed on day 18 as indicated by the termination of the lines. As zero values cannot be plotted on logarithmic scales, values of one (1.0E+00) haemoplasma copy per µl blood (and qPCR reaction) are treated as the equivalent of zero on this and subsequent graphs within this chapter.*

None of the mice had haemoplasma DNA detected in pre-infection blood samples before entering the experiment. Both mice inoculated with the *M. haemomuris*-like haemoplasma isolate amplified the organism to 3.00E+05 and 1.17E+06 genome copies per µl blood within 4 days post inoculation. Haemoplasma genome copies then dropped sharply (1.25E+03 and 3.38E+03 per µl respectively) on day 7 and mice were exsanguinated for future inocula on day 8 of the experiment. Mice inoculated with the *M. haemomuris* isolate showed a later amplification (reaching 4.05E+03 and 1.33E+04 haemoplasma genome copies per µl blood on day 7) and also were exsanguinated on day 8. *Eperythrozoon coccoides*-inoculated mice showed a more erratic pattern of amplification with the first mouse only amplifying the organism to 25 haemoplasma genome copies per µl blood on day 18 post inoculation, and the second mouse exsanguinated on day 18 of the experiment whilst harbouring 2.67E+03 haemoplasma genome copies per µl blood.

The two mice inoculated with *M. suis* had detectable *M. suis* genome copies by qPCR only within the first week of the experiment with maximums of 14 and 9 *M. suis* genome copies per µl blood reached on day 7 respectively.

2.3.3.2 Initial passage of rodent haemoplasmas into laboratory rats

2.3.3.2.1 Clinical signs and post-mortem examinations

No clinical signs of ill health were noted in any rats throughout the experiment. As rats were rehomed following the experiment, no post-mortem examinations were carried out.

2.3.3.2.2 Haemoplasma blood copies in rat blood samples

Normalised relative haemoplasma (*M. haemomuris*, *M. haemomuris*-like haemoplasma and *E. coccoides*) genome copies per µl blood after inoculation into naïve Wistar rats are shown in **Figure 12**.

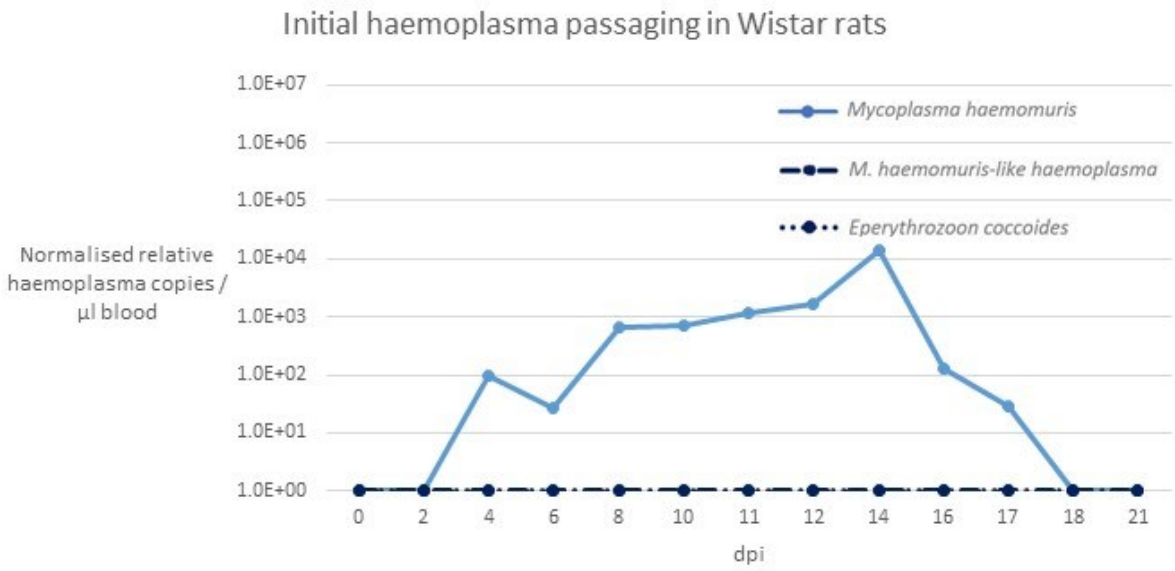


Figure 12 *Haemoplasma genome copies during initial passaging into Wistar rats. Days post inoculation are plotted on the x-axis and normalised relative haemoplasma genome copies per µl blood are plotted on the y-axis. Connected dots represent individual rats; solid light blue line: M. haemomuris; dashed blue line: M. haemomuris-like haemoplasma; dotted blue line: E. coccoides.*

None of the three rats had haemoplasma DNA detected in their pre-infection blood samples, before entering the experiment. One rat, inoculated with *M. haemomuris* amplified the organism to 1.42E+04 genome copies per µl blood on day 14 post inoculation, followed by a rapid decline until becoming undetectable by day 21 post inoculation. The remaining two rats, inoculated with *M. haemomuris*-like haemoplasma and *E. coccoides* containing blood samples, did not have detectable haemoplasma DNA in their blood at any time point tested.

2.4 DISCUSSION

2.4.1 Identification of wild-caught rodent haemoplasma isolates

Five rodent haemoplasmas were successfully isolated from wild-caught field mice (*Apodemus* spp.). Based on near-complete 16S rRNA gene sequences, these were identified as *M. haemomuris* (two isolates, both 99% identity to published *M. haemomuris* 16S rRNA gene sequences), *E. coccoides* (one isolate, 97% identity) and two previously unnamed rodent haemoplasmas that failed to reach the 97% threshold of 16S rRNA gene sequence identity with a named species (283). Identification and phylogeny based on a 464 base pair PCR amplicon from a variable region within the 16S rRNA gene was only extended to include further sequence data for three of the five isolates that were considered for further passaging into laboratory rodents to establish a model of *M. haemofelis* infection with a phylogenetically related haemoplasma species. This lack of obtaining further sequence data for the remaining isolates potentially delayed the discovery and description of a novel species within the rodent haemoplasmas which is especially true for isolate #9 that did not show significant similarities with any named haemoplasma species.

Haemoplasma phylogeny based on 16S rRNA gene sequences alone lacks resolving power due to this gene being highly conserved (26, 31), and traditionally phylogeny has been expanded to include other ribosomal genes such as *rnpB* or the ITS (17, 264) to give more reliable data on the relatedness of the species being analysed. The threshold of 97% identity using 16S rRNA gene sequence has also been recently revised with a more stringent threshold of 99% suggested for bacterial species-determination (19). Using this 99% threshold, only the *M. haemomuris* isolate #6 can successfully be described as identified, whereas the *E. coccoides* isolate #18 might still comprise a novel rodent haemoplasma species. As an alternative to 16S rRNA gene sequences, protein-coding gene sequences can be used for high-resolution phylogeny as they tend to be less conserved at a nucleotide level than ribosomal RNA genes. However, there are associated difficulties in obtaining such sequences from haemoplasmas in the first place due to the difficulties in amplifying more variable regions (16). Evolutionary reconstructions based on such expanded sequences can limit construction of phylogenetic trees as only those species for which sequence data are available can be included. At the time of the studies described in this thesis, whole genome sequences were available for nine haemoplasma species (1.1) but these did not include any rodent haemoplasmas nor more recently reported haemoplasma species/isolates within the HF-group of haemoplasmas, such as '*Ca.* M. haemohominis', '*Ca.* M. haemomacaque' or HF-group haemoplasmas found in wild carnivores, such as wolves and foxes (65). Our approach in the current study focused on phylogenetically defining the novel haemoplasma isolates derived in this study within the published 16S rRNA gene and ITS sequences available from other rodent haemoplasmas from previous studies, which was especially true for the *M. haemomuris* isolates.

The ITS region, between the 16S and 23S rRNA genes, is proposed to be unique to each bacterial strain ("ribotype") and commonly used in clinical diagnostics to monitor outbreaks (284). The high variability of this region may provide a better means of defining new species than 16S rRNA gene phylogeny alone, and has been explored for Mycoplasmas (282). Based on ITS nucleotide sequence comparisons, isolate #6 (considered to be *M. haemomuris)* had 93% identity to '*Ca.* M. haemomuris subspecies ratti' and 90% identity to '*Ca.* M. haemomuris subspecies musculi'. This compares to 99% identity to both of these proposed subspecies based on 16S rRNA gene sequences. It is difficult to determine whether our isolate defines a novel species or simply a novel strain from a geographically different location. *Mycoplasma haemofelis* and *M. haemocanis* are two distinct haemoplasma species that share 99% identity based on their 16S rRNA gene sequences (196) and 94% identity in the ITS. This 94% figure represents a marginally higher percentage identity than shown (93%)

between our study's *M. haemomuris* isolate and its nearest phylogenetic neighbour '*Ca.* M. haemomuris subspecies ratti'. Thus our *M. haemomuris* isolate may represent a novel haemoplasma species. Unfortunately, no ITS sequence data could be obtained for isolate #18 *E. coccoides* nor was it available for other *E. coccoides* isolates in the database nor its nearest phylogenetic neighbour '*Ca.* M. turicensis'. The relationships within this HF-group of haemoplasmas, that include rodent and non-rodent (including feline) hosts, remains underexplored but might be crucial to understanding haemoplasma species-specificity (or lack thereof) amongst closely related isolates.

More sequence data, ideally non-ribosomal gene sequences, will be required to further assess the phylogenetic relationships within the rodent haemoplasmas and determine if our novel *M. haemomuris, E. coccoides* and other isolates are indeed all members of their respective species, especially when they are isolated from different host species and different geographical locations. However, for the purpose of the current thesis and further studies described in later chapters, isolate #6 will be referred to as *M. haemomuris,* isolate #18 as *E. coccoides* and isolate #13 as *M. haemomuris*-like haemoplasma.

2.4.2 Successful passage of wild-caught rodent haemoplasma isolates into laboratory mice

Using quantitative methods of detection (qPCR), two of two inoculated mice successfully became infected with *M. haemomuris* as demonstrated by a rapid increase in haemoplasma genome copies per µl of blood over three days, from being undetectable on day 4 to over 1.00E+05 on day 3 post inoculation. Comparably rapid *in vivo* haemoplasma amplification was seen in two of two mice inoculated with *M. haemomuris*-like haemoplasma, although it was followed by an equally rapid decline in the observation period (**Figure 11**).

The two mice inoculated with *E. coccoides* showed a different pattern of amplification with relative haemoplasma genome copies per µl of blood only reaching a maximum of 1.22E+04 and likely bordering the limit of detection of the PCR assay at other timepoints. In brief, the limit of PCR assay detection of haemoplasmas can be explored by considering that DNA was extracted from a maximal volume of 10µl of blood, as no more than this could be collected safely once daily from the laboratory mice over a prolonged period of time. The DNA from this aliquot of blood was eluted into 50µl of elution buffer during extraction and then only 5µl of this eluate included in each qPCR assay (2.2.1.2). Thus, effectively qPCR is being performed (assuming 100% efficiency of DNA extraction) on a sample equivalent to 1µl of blood. To reliably detect every haemoplasma copy in a blood sample, 10 replicates (i.e. the entire eluate) would have been required. Low haemoplasma genome copy numbers (less than 1000 per ml of blood) have been reported in other studies using larger blood sample volumes (103, 120, 131, 149, 285) but were not feasible for us to report as laboratory mice are small (approximately 20g in the used age-range) and their blood volume is 58.6ml per kg, meaning there is approximately 1.16ml of blood in an average laboratory mouse. This illustrates the dilemma of being unable to reliably detect low haemoplasma copy numbers and explain the apparent fluctuation between non-detectable and relatively high (over 1000 per ml blood) copy numbers that was seen in infected mice. This low-level fluctuation does not necessarily reflect true haemoplasma copy cycling that is seen in cats or pigs when using much larger sample volumes and changes in haemoplasma copy numbers (107, 120). This might also explain a potential 'lag-phase' that could represent the presence of low copy numbers that remained undetected.

Peaks and troughs of haemoplasma genome copies in the bloodstream could have been missed in the current study due to blood sampling being conducted at intervals of a few days apart. This blood sampling schedule was adopted primarily due to an inability to collect tiny blood volumes from mice more frequently initially, but practical experience derived in the current studies meant that daily

blood sampling was thought to be possible in subsequent studies. Potential peaks (or troughs) of blood haemoplasma copy numbers in the laboratory mice were also possibly missed by the killing of the *M. haemomuris* and *M. haemomuris*-like haemoplasma inoculated mice at day eight post inoculation, which was soon after amplification had been confirmed by increasing haemoplasma copies per µl of blood over time. Exsanguination of infected mice was, however, deemed the most effective way of securing cryopreserved inoculate for use in future studies, given that only one original sample was obtained from each individual field mouse (2.2.1.1.3.1).

The successful amplification of rodent haemoplasmas in laboratory mice in our study was not entirely unexpected due to previous reports of successful passaging of haemoplasmas from sources similar to ours, such as *M. haemomuris* infection of Balb/C mice from samples of infected *Apodemus argenteus* specimens (197). However, as outlined in 2.4.1, confirmation of the species used in such passaging experiments was not extended beyond ribosomal gene phylogeny, so accurate predictions of host species and haemoplasma species specificity were not available.

2.4.3 Failure to passage *M. suis* into laboratory mice

In the two mice inoculated with porcine blood containing *M. suis*, hemoplasma genome copies were only detected in blood sampled on days four and seven post inoculation. Most importantly, the blood haemoplasma genome copies per µl blood (1 to 14 copies) at these time points stayed below the level that would have been expected through the dilution of the inoculum in the individual mouse's blood volume alone of 7.43E+07 *M. suis* copies per µl blood. This figure is derived from knowing that the total blood volume of a 20g mouse is approximately 1.16ml and 8.69E+10 *M. suis* copies were inoculated into each mouse. Assuming complete uptake of the inoculated *M. suis* into the murine blood stream, there would be 7.43E+07 *M. suis* copies per µl blood detectable. Whilst this cannot fully explain the increase seen between days four and seven, one might speculate that uptake from the intraperitoneal injection site was delayed or, alternatively, that the haemoplasmas were able to briefly amplify before being eliminated by the murine immune system. However, it is probably more likely that the variation between days four and seven post inoculation is due to stochastic variation and may not have been apparent if more replicate qPCRs had been performed or a larger number of mice had been used. Our observation that *M. suis* could not establish infection in healthy laboratory mice concurs with observations by German research groups who were able to detect *M. suis* after intraperitoneal inoculation into four-week-old OF-1 mice (Oncins-France-1, an outbred strain) but were unable to establish infection or detect *M. suis* DNA after the first week (217). It can only be speculated how reliable the claims by Chinese research groups are that they have a mouse model of *M. suis* infection established in an outbred mouse strain; Kunming mice. (248, 249, 286). Full methodologies or nucleotide sequences for phylogenetic comparison are not available but using immunocompromisation (by the use of high doses of glucocorticoids) and repeated inoculation of *M. suis* (at least every three days) in combination with non-quantitative, gel-based methods of PCR detection does question the reliability of their model (287). Currently we have found no evidence to support successful *M. suis* growth outside of their natural porcine hosts. Future research might involve different strains of mice, including genetically modified strains such as athymic mice, to assess species-specific infection of erythrocytes versus species-specific immune evasion of the organism.

2.4.4 Successful passage of *M. haemomuris* and failure to passage *E. coccoides* and *M. haemomuris*-like haemoplasma into laboratory rats

The *M. haemomuris* isolate #6 successfully established infection in a ten-week-old, SPF, female Wistar rat. No haemoplasma DNA was detected in the rats each inoculated with *E. coccoides* and *M. haemomuris*-like haemoplasma throughout the observation period. However, we did not perform

any qPCR on splenic tissue from the remaining two rats, which may have demonstrated latent infection at a later stage of the experiment, similar to what was seen in previous rat experiments on days ten and fifteen post inoculation of *M. haemomuris*-containing inocula (197).

Whilst this report of *M. haemomuris* infection in a laboratory rat is limited by its demonstration in only one animal, it is the first time that haemoplasma genome copy numbers have been monitored in a rat over the course of experimental infection using qPCR. However, the *M. haemomuris* copies per µl of blood detected in the rat were much lower than those detected in the mice, with a maximum of 1.42E+04 copies per µl blood being reached two weeks post inoculation (**Figure 12**) in the rat compared to the mice which showed a 100-fold higher level of *M. haemomuris* copies per µl blood just one week post inoculation (**Figure 11**) with a potential peak still to be reached. It could be speculated that our *M. haemomuris* isolate underwent changes during its passaging in laboratory mice that subsequently compromised its ability to establish infection in rats. Another possibility is that the *M. haemomuris* isolate is able to evade the mouse immune system better than the rat immune system, as the infection kinetics seen in rats (short-lived bacteraemia) was compatible with that of an innate immune response clearing infection (288). Whilst rats would offer a larger laboratory model of haemoplasma infection compared to mice, the observed preliminary infection kinetics seen for *M. haemomuris* did not resemble the infection kinetics seen for the primary pathogenic haemoplasma species in either cats or pigs regarding magnitude of haemoplasma bacteraemia or duration of infection with pathogenic haemoplasmas (90, 107, 120). Additionally, the use of rats would be associated with higher costs and less well-established laboratory reagents being available compared to mice. Thus, mice were considered to be a more suitable host for a rodent model of haemoplasma infection and so inoculation of *M. haemomuris* into laboratory rats was not further explored in the current studies.

2.4.5 Limitations of the current study and suggested future work using the novel haemoplasma isolates

We have been unable to extend the phylogenetic classification and identification of our newly obtained haemoplasma isolates to subspecies-level, and we are currently unsure as to whether they represent novel strains or novel species of haemoplasmas when applying the most stringent criteria of classification. Future research should try to include sequencing of rnpB, DNAK and GAPDH for these isolates, as these genes have been successfully used for haemoplasma phylogeny in previous studies (216, 289). Ideally, however, future research would include derivation of whole genome sequences for our, and other rodent, haemoplasma isolates, to define phylogeny more accurately. Recently, cost reductions in bacterial genome sequencing have opened up this possibility to researchers working on less 'funded' pathogens, but the technique still requires adequate amounts of sufficiently pure template DNA to be available for sequencing. Currently, this DNA cannot easily be obtained for haemoplasmas that cannot be grown outside the murine host because if they are grown within the murine host, purification from host mouse DNA is difficult to achieve (94). Several hundred mice would also likely be required to provide sufficient haemoplasma DNA for purification attempts, and so this would be a significant undertaking and outside of the scope of the current experiments. In the future whole rodent haemoplasma gene sequencing might become more feasible if sequencing methodology improves or if haemoplasma *in vitro* cultivation is successful. An established mouse model of haemoplasma infection to act as a source of viable haemoplasmas for *in vitro* experiments will also be important.

Due to the pilot nature of the *in vivo* infection experiments described in this chapter, a full rodent model of haemoplasma infection was not established in these studies, but the rodent experiments represented the foundations of such a model as we were able to successfully establish infection in

laboratory mice and rats and develop suitable means of cryopreservation, inoculation and monitoring of haemoplasma copies in inoculated rodents. Model selection and subsequent establishment of a repeatable model of infection are important. One should also consider the model's analogy to *M. haemofelis* infection, as if the aim is to establish an animal model of disease, it ideally needs to represent its closest phylogenetic neighbour that is a primary pathogen with significant morbidity i.e. *M. haemofelis*.

Future work will focus on repeating *M. haemomuris* and *E. coccoides* inoculations and infection in C57BL/6 mice and concentrate on the monitoring of infection kinetics. This will be achieved by more frequent sampling to evaluate more thoroughly the potential fluctuations of haemoplasma copies per µl blood, which represents an unexplained feature of *M. haemofelis* infection in cats. Ultimately, protection from haemoplasma reinfection and cross-protection can be evaluated in a mouse model that resembles *M. haemofelis* infection in cats as closely as possible.

3 ESTABLISHMENT AND INITIAL CHARACTERISATION OF A MOUSE MODEL OF HAEMOPLASMA INFECTION

3.1 INTRODUCTION

3.1.1 Known characteristics of rodent haemoplasma infection

The kinetics of rodent haemoplasma infection are largely unknown. Similarities to feline or porcine haemoplasma infection (cats and pigs being the veterinary host species most investigated, and considered important, to suffer from clinical haemoplasmosis) would be required to successfully select a suitable mouse model of haemoplasma infection for further studies. Early studies following rodent haemoplasma infection over time were based on microscopy, and hence are likely to have missed low-level bacteraemia. Eliot (238) reported *E. coccoides* to appear two to four days post splenectomy in mice, with a dramatic increase in numbers of parasitized red blood cells over two to three days and less pronounced peaks of bacteraemia at intervals of several days afterwards, with the bacteraemia sometimes being still detectable three months after splenectomy. Similar observations of haemoplasmas becoming visible approximately three days post inoculation (dpi) or after splenectomy, with fluctuating levels and potentially deadly relapses over the first two to three weeks, were made by Ford and Eliot (237) in rats infected with *M. haemomuris.* These authors also reported a drop in viability of the organism following transfer into other healthy animals, once peak bacteraemia (at two to five days) had passed. Marmorston-Gottesman and Perla (240) reported that *M. haemomuris* (referred to as *Bartonella muris*) was detectable on red blood cells three to six days after infection of immunocompetent young white mice, with anaemia developing at around five dpi. Rikihisa, Kawahara (197) reported an increase in splenic weight at necropsy in mice experimentally infected with *M. haemomuris* (referred to as *Haemobartonella muris*) with a peak around five dpi that gradually decreased, but did not revert to normal, until the end of the experiment at 25 dpi. In this study, stained blood smears were said to be positive for haemoplasmas at ten dpi, but no further smears were taken. More recently, Cohen, Shemesh (34) monitored the infection dynamics of a '*M. haemomuris*-like haemoplasma' in wild gerbils using the generic haemoplasma assays (185) described in Chapter 2 to monitor their successful passage into laboratory mice. Their findings included a peak in haemoplasma genome copies per µl blood occurring around 25 to 45 dpi, followed by a decline and plateau phase from 65 to 400 dpi (representing the end of the experiment) suggesting that persistent infection is common (34).

3.1.2 Protective immunity against haemoplasma infection

Haemoplasma infection can be chronic and difficult to eradicate from cats, pigs and mice (89, 120, 136, 252). Until recently, no treatment protocol had been described that claimed to establish haemoplasma elimination (135), supporting the need for a vaccine if the aim is protection from infection and thus future relapses. However, despite promising immunoreactive *M. suis* antigens being investigated as vaccine candidates in pigs (120), no effective vaccine has been developed for any haemoplasma species to date. Protective immunity in animals that have recovered from haemoplasma infection would drive investigations into vaccine candidates, although it is not a strict requirement in vaccinology (290).

In cats, protective immunity against re-infection with the same haemoplasma species has been demonstrated in small pilot studies for *M. haemofelis* and '*Ca.* M. turicensis' (107, 124). In mice, Ford and Eliot (237) made a case against protective immunity as they documented relapses and anaemia in splenectomised rats previously 'recovered' from *M. haemomuris* infection; however they

were not aware of the role of the spleen in linking the innate and adaptive immune response (291) or more specifically, the role of splenic macrophages in clearing blood-borne pathogens such as haemoplasmas (292). Splenectomy has since been used to model more severe haemoplasmosis in piglets (120) and been suggested to be a risk factor in canine haemoplasmosis (293) but this observation regarding splenectomy does not rule out that protective immunity in non-splenectomised animals to haemoplasmosis can occur. The fact that co-infections with different haemoplasma species can occur in the same host (98, 100, 294) suggests that cross-protection is very unlikely. There is some evidence in cats that previous exposure to different haemoplasma species, investigated with '*Ca.* M. turicensis' and '*Ca.* M. haemominutum', might even enhance clinical disease from subsequent *M. haemofelis* infection (180, 295).

3.1.3 PCR detection and monitoring of infection kinetics of haemoplasmas

Monitoring of infection kinetics requires quantitative methods to differentiate low-level chronic infection from fulminant bacteraemia, and to compare bacterial loads across timepoints and species as required for assessment of a rodent model of haemoplasma infection. The use of a generic haemoplasma qPCR assay for quantification can only be used with confidence when only a single haemoplasma species is present i.e. multiple-infections cannot be quantified by a generic qPCR as differentiation of species is not possible. Cohen, Shemesh (34) submitted 20% of their generic haemoplasma qPCR amplicons for Sanger sequencing to determine the haemoplasma species present, whilst monitoring '*M. haemomuris*-like haemoplasma'-infection dynamics, to help ensure the presence of only one haemoplasma species (the '*M. haemomuris*-like haemoplasma' species). This method would not be suitable for experiments involving two or more haemoplasma species as sequencing of qPCR amplicons might still miss infection with more than one species. Amplification of one haemoplasma species with a significantly lower number of copies in the bloodstream is likely to be outcompeted by another species that is present at higher copies.

In some host species, where co-infection with more than one haemoplasma species occurs, the problem of quantification of multiple infecting haemoplasma species has been solved by developing species-specific conventional PCR assays (296) and subsequently qPCR assays (187, 297, 298) designed to target regions of variance in the 16S rRNA gene, that otherwise exhibits a high degree of conservation across species. In cats, specific qPCR assays are available for *M. haemofelis*, '*Ca.* M. turicensis' and '*Ca.* M. haemominutum' (38, 297) with no observed cross-reactivity between the respective feline haemoplasma species at 1.0E+06 16S rRNA gene copies of the non-target haemoplasma species per reaction. In dogs, *M. haemocanis* and '*Ca.* M. haematoparvum' can be detected by species-specific qPCR assays and no cross-reaction occurs up until a concentration of 1.0E+05 16S rRNA copies of the non-target haemoplasma species per reaction (187).

The 16S rRNA gene is used for species identification as it is highly conserved within, and in parts even across, species (299). The feline haemoplasma *M. haemofelis* is 84 % identical to '*Ca.* M. haemominutum' and 88 % identical to '*Ca.* M. turicensis' based on 16S rRNA gene sequencing (38). Interestingly, the *M. haemofelis* 16S rRNA gene sequence is identical to the corresponding sequence of the canine haemoplasma *M. haemocanis*, and '*Ca.* M. haemominutum' shares 94% 16S rRNA gene identity with the canine haemoplasma '*Ca.* M. haematoparvum', despite these organisms infecting different host species (298). The qPCR assays used for the detection of *M. haemofelis* and '*Ca.* M. haemominutum' (297) have been successfully used to detect related haemoplasma species when used on a different host, i.e. detection of '*Ca.* M. haematoparvum' in a dog using a feline '*Ca.* M. haemominutum' qPCR assay (298), and detection of *M. haemocanis* in a dog using a feline *M. haemofelis* qPCR assay (148). Our two recently derived rodent haemoplasma isolates of *M. haemomuris* and *E. coccoides* (described in Chapter 2) are 89% identical based on 16S rRNA gene

sequencing and without significant insertions/deletions that could easily be used for sequence differentiation. High similarities with evenly spaced nucleotide polymorphisms pose a challenge to species-specific qPCR design that will not result in cross-reactions, as would be required for any experiments assessing cross-protection (i.e. infection with one haemoplasma species protecting against infection with another haemoplasma species).

Quantitative PCR assays for the feline and canine haemoplasma species have been duplexed with a feline and canine internal control qPCR, respectively, to ensure the ability to detect amplifiable DNA and an absence of signficant qPCR inhibitors (100, 187). These multiplexed quantitative PCR assays also offer a means of haemoplasma copy number normalisation against the amount of host DNA present in a given blood sample (297).

3.1.4 Objectives

The first objective of this chapter was to develop qPCR assays specific to the two rodent haemoplasma species (*M. haemomuris* and *E. coccoides*) described in successful mouse inoculation studies in Chapter 2, and use them to monitor the infection kinetics of *M. haemomuris* and *E. coccoides* in C57BL/6 mice following experimental infection. The second objective was to further assess whether these two rodent haemoplasma species could be used to establish a suitable surrogate model of haemoplasma infection in cats (or other veterinary species) and assess whether protection from re-infection or cross-protection occurs.

3.2 MATERIALS AND METHODS

3.2.1 Development of qPCR assays to specifically detect *M. haemomuris* and *E. coccoides* DNA in mouse blood samples

3.2.1.1 Development of a M. haemomuris qPCR singleplex assay

3.2.1.1.1 Origin of *M. haemomuris* 16S rRNA gene template DNA

Template DNA for the *M. haemomuris* 16S rRNA gene qPCR assay development was generated from the pilot mouse infection experiments described previously (see 2.2.3). In brief, DNA was extracted using the NucleoSpin® Blood kit (Macherey-Nagel) and eluates stored at -20°C until use. As the *M. haemomuris* qPCR assay development overlapped with the first part of the experiment describing primary infection with *E. coccoides* and *M. haemomuris* (see 3.2.2), samples from these studies were also utilised. Where indicated, mouse DNA was used as a negative control or as 'background' DNA and was derived from pre-infection screenings from previous and ongoing mouse studies that had generated negative qPCR results following screening with a generic haemoplasma qPCR (2.2.3.3).

As no concentrated genomic *M. haemomuris* 16S rRNA gene template DNA was available to use for studies requiring a high concentration of template DNA (i.e. to produce serial dilutions for standard curve generation) amplicons derived from primers 313-HBT-F and 680-HF-group-R (which amplified a section of 595 base pairs on the haemoplasma 16S rRNA gene) were generated as described in 2.2.2.1.2 and diluted 1 in 1,000,000 with elution buffer (BE, Macherey-Nagel) prior to use, unless noted otherwise. For each experiment, amplicons were prepared and diluted individually.

3.2.1.1.2 *M. haemomuris* qPCR primers and probe design

Mycoplasma haemomuris qPCR primer pairs and TaqMan probe were designed to the partial 16S rRNA gene consensus sequence generated in 2.2.2.1.2. A multiple sequence alignment of the 16S rRNA gene sequences of the novel *M. haemomuris* and *E. coccoides* isolates Langford #6 and #18 and other closely related HF-group haemoplasmas was generated in Mega7 (268) and assessed for divergent regions. Other isolates included the '*M. haemomuris*-like haemoplasma' isolate #13 and published sequences of *E. coccoides* (AY171918.1, EF175168.1, EF175169.1, EF175170.1), *M. haemomuris* (AB918692.1, AB758434.1, AB758435.1, AB758439.1, AB758440.1), '*Ca.* M. haemohominis' (GU562823.1) and '*Ca.* M. turicensis' (DQ464418.1, KR905459.1, KM275267.1). Primers and probes were designed using Primer3Plus (300) in "primer list" mode on the *M. haemomuris* 16S rRNA gene sequence to select primers spanning divergent regions, preferably being most divergent between species at the 3' end and conserved within multiple isolates of the same species. Primer generation was achieved using the following settings:

Primer size (base pairs): minimum 16; optimum 20; maximum 27
Primer Tm (°C): min. 58.0; opt. 60.0; max. 62.0
Primer G+C%: min. 40; max. 60
Maximum self-complementarity: 4
Maximum 3'-self-complementarity: 1
Maximum poly-X: 4
Mispriming (rodent and simple): 12

Primer pairs were selected, aiming for amplicon sizes of 100 to 250 base pairs and subsequently assessed in "detection mode", including designing internal hybridisation probes with the following settings:

Probe size (base pairs): minimum 18; maximum 27
Probe Tm (°C): min. 68.0; opt. 70.0; max. 72.0
Probe G+C%: min. 20; max. 80

Predicted amplicons were assessed for secondary structure formation using the Mfold webserver (301) and primers discounted if secondary structure at the primer-binding site was predicted to have a melting temperature above the primer melting temperature, which would have interfered with primer annealing. The TaqMan probe was labelled with Texas Red (TEX) at the 5'-end and with black hole quencher 2 (BHQ2) at the 3'-end. All oligonucleotides (primers/probe) were ordered from Metabion, Germany.

3.2.1.1.3 Optimisation of *M. haemomuris* qPCR conditions using SYBR Green 1

3.2.1.1.3.1 QPCR conditions and dissociation curve analysis

Six primer pairs (designed as above; shown later in 3.2.1.1.2 and 3.2.1.2.2) were assessed for their ability to amplify the *M. haemomuris* 16S rRNA gene. Each qPCR consisted of 2X GoTaq (Promega), 4.5mM $MgCl_2$, SYBR Green 1 (1 in 100,000; Sigma-Aldrich Ldt., Poole, UK) and forward and reverse primers, each at 200nM final concentration. Two microlitres of template DNA and water were added to a final volume of 25µl. Template DNA for the initial reactions was derived from pooling DNA samples from primary passages of novel rodent haemoplasmas (3.2.1.1.1) at a final concentration of approximately 2.5E+03 genome copies per µl. Each qPCR was run in duplicate and negative controls (water and mouse DNA) were included for each primer pair.

All qPCRs were performed on a Mx3005 qPCR thermal cycling system (Agilent) under the following conditions: Initial incubation for 2 mins at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. Fluorescence was detected at 516nm at the end of each annealing/extension phase. This was followed by a dissociation curve spanning 70°C to 90°C in 1°C increments.

SYBR Green 1 reports the presence of double-stranded DNA as it fluoresces stronger when intercalated within double-stranded DNA. After initial detection (by reporting of the threshold cycle value) of amplifiable DNA, the melting temperature of the PCR amplicon (dependent on a number of factors including the length and GC-content) can be determined by a measuring fluorescence over a temperature gradient; the detection of a sudden drop in fluorescence signifies dissociation of double-stranded DNA and can be used to confirm the presence of a single amplicon (302).

3.2.1.1.3.2 Assessment of M. haemomuris singleplex assay cross-reactivity to E. coccoides attributed to primers

Initial assessment of cross-reactivity was carried out on two primer pairs that were tested against *E. coccoides* genomic DNA (Ct 26.06 when using the HF-assay described in 2.2.1.2.2; equivalent to 1.62E+04 genome copies per reaction) in triplicate qPCRs. Positive (*M. haemomuris* genomic DNA*)* and negative (water) controls were included. Reaction conditions of each qPCR, including the subsequent generation of a dissociation curve, were identical to the ones described above (3.2.1.1.3.1).

3.2.1.1.3.3 Reaction efficiency evaluation (standard curve generation)

Reaction efficiency was assessed for two primer pairs, using a series of dilutions of target DNA consisting of *M. haemomuris* 16S rRNA gene PCR amplicons, spanning the target region. The amplicons were generated as described above (3.2.1.1.1) followed by a series of ten-fold dilutions in a mouse DNA background, comprising DNA from haemoplasma-free mice tested during the pre-

infection screening process (2.2.3.3). The dilution series was prepared immediately prior to use and run in duplicate on the same qPCR plate, using the conditions described in 3.2.1.1.3.1.

3.2.1.1.3.3.1 Calculating assay efficiency from the slope of the amplification curve

A graph of Ct versus $\log_{10}$ of the copy number of the template was produced, and the slope of this graph was used to calculate qPCR efficiency using the MxPro™ v4.10 software package (Agilent) and the following formula:

$$E = 10^{(-1/\text{slope})} - 1$$

where E = qPCR efficiency and the slope being defined as the relative increase of Ct values over each 10-fold dilution of template.

As described earlier (2.2.1.2.2.2), PCR amplification follows an exponential curve that doubles the amount of product (amplification equal to 2^{Ct}) with each cycle. As Ct values were plotted against $\log_{10}$ of the copy number (amplification being equal to $10^{-1/\text{slope}}$), a slope of -3.32 is considered ideal and equates to 100% efficiency, due to $10^{-1/-3.32} = 2$ and $10^{-1/-3.32} - 1 = 1$ (100%).

3.2.1.1.4 Analysis of qPCR data

Analysis was carried out using the MxPro™ v4.10 software package (Agilent). Threshold fluorescence was set at 300 relative fluorescence units (Rf) for all assays (*M. haemomuris*-specific, *E. coccoides*-specific, and when in use, the host internal control qPCR assay). Data were compared using threshold cycle (Ct) values. For the purpose of assay development, no normalisation of Ct values was carried out. Where relative haemoplasma genome copy numbers are reported, these were calculated using the formula given in 2.2.1.2.2.2.

3.2.1.2 Development of an E. coccoides qPCR singleplex assay

3.2.1.2.1 Origin of *E. coccoides* 16S rRNA gene template DNA

Template DNA for qPCR assay development was generated from the pilot mouse infection experiments (see 2.2.3) described previously and was extracted and stored as described for the *M. haemomuris* singleplex assay development (3.2.1.1) but using samples containing *E. coccoides* genomic DNA.

As no concentrated *E. coccoides* genomic DNA was available, amplicons from primers 313-HBT-F and 680-HF-group-R were generated as described in Chapter 2 and 3.2.1.1.1 and diluted 1 in 1,000,000 with elution buffer (BE, Macherey-Nagel) prior to use, unless noted otherwise. Amplicon dilutions were prepared individually for each experiment and used for studies requiring a high concentration of template 16S rRNA gene DNA.

3.2.1.2.2 *E. coccoides* qPCR primers and probe design

Eperythrozoon coccoides qPCR primer pairs and a TaqMan probe were designed to the partial 16S rRNA gene consensus sequence generated in Chapter 2 in an identical approach as outlined for the *M. haemomuris* singleplex assay (3.2.1.1.2) using Primer3Plus (300). Primer pairs that produced predicted secondary structure by MFold (301) were excluded and all oligonucleotides (primers/probes) were ordered from Metabion, Germany.

3.2.1.2.3 Optimisation of *E. coccoides* qPCR conditions using SYBR Green 1

3.2.1.2.3.1 QPCR conditions and dissociation curve analysis

Four primer pairs were assessed for their ability to amplify the *E. coccoides* 16S rRNA gene sequence. Reaction conditions for each qPCR were as described for *M. haemomuris* above (3.2.1.1.3.1) using

primers designed to the *E. coccoides* 16S rRNA gene. Template DNA for the initial reactions was derived from pooling DNA samples from primary passages of novel rodent haemoplasmas (3.2.1.2.1) at a concentration of approximately 3.0E+03 genome copies per µl. Negative controls (water and mouse background DNA) were included and qPCR conditions, including thermal cycling conditions and dissociation curve generation, were identical to the procedures outlined in 3.2.1.1.3.1.

3.2.1.2.3.2 Assessment of E. coccoides singleplex assay cross-reactivity to M. haemomuris attributed to primers

Initial assessment of cross-reactivity was carried out on three primer pairs, challenged with genomic *M. haemomuris* template DNA (Ct 16.30 when using the HF-assay described in 2.2.1.2.2; equivalent to 1.45E+07 genome copies per reaction) in triplicate qPCRs, with positive (*E. coccoides*) and negative (water) controls. Reaction conditions and generation of dissociation curves was identical to the method described for *M. haemomuris* above (3.2.1.1.3.1).

3.2.1.2.3.3 Reaction efficiency evaluation (standard curve generation)

Reaction efficiency was assessed for two primer pairs, using a series of dilutions of target DNA consisting of *E. coccoides* 16S rRNA gene PCR amplicons, spanning the target region. The amplicons were generated as described above (3.2.1.1.1) followed by a series of ten-fold dilutions in a mouse background DNA, comprising DNA from haemoplasma-free mice tested during the pre-infection screening process (2.2.3.3). The dilution series was prepared immediately prior to use and run in duplicate on the same qPCR plate, using the conditions described in 3.2.1.1.3.1.

3.2.1.2.3.3.1 Calculating assay efficiency and slope of amplification curve

Assay efficiency and slope of the amplification curve were calculated as described for 3.2.1.1.3.3.1.

3.2.1.2.4 Analysis of qPCR data

Data analysis was carried out as described in *3.2.1.1.4*.

3.2.1.3 Duplexing rodent haemoplasma and host DNA qPCR assays

3.2.1.3.1 Novel rodent haemoplasma and internal mouse host control (GAPDH) qPCR primers and probe

Mycoplasma haemomuris and *E. coccoides*-specific primers and probes, as designed in 3.2.1.1.2 and 3.2.1.2.2, were used to develop haemoplasma species-specific qPCR singleplex assays using a TaqMan probe. Primers and probe from a previously published qPCR assay targeting the cat GAPDH gene (261) was used as an internal control as previously described under singleplex assay conditions, as the respective primer binding sites of the mouse GAPDH gene are identical to the cat GAPDH gene (2.2.1.2.2). The TaqMan probes specific to the *M. haemomuris* and *E. coccoides* 16S rRNA genes were labelled with Texas Red (TEX) at the 5'-end and a BHQ2 at the 3'-end and synthesized by Metabion. The TaqMan probe targeting the mouse GAPDH gene was labelled with 6-carboxyflourescein (FAM) at the 5'-end and a black hole quencher 1 (BHQ1) at the 3'-end and synthesized by Invitrogen, UK.

3.2.1.3.2 Optimisation of duplex qPCR conditions (*M. haemomuris* and *E. coccoides*)

3.2.1.3.2.1 Singleplex qPCR conditions for the M. haemomuris and E. coccoides assays using a TaqMan probe

Initial singleplex qPCR conditions consisted of 2X GoTaq (Promega), 4.5mM $MgCl_2$, forward and reverse primers (specific to either *M. haemomuris* or *E. coccoides* 16S rRNA genes) each at 200nM and a TaqMan probe at 200nM final concentration. Probe concentration was subsequently varied at

200nM, 100nM and 50nM during optimisation. Water and template DNA were added to a final volume of 25µl. Initial singleplex reactions were tested with *M. haemomuris* and *E. coccoides* 16S rRNA gene amplicon dilutions (3.2.1.1 and 3.2.1.2.1) at three different concentrations ("low", "medium" and "high") in mouse genomic DNA generated from pre-infection screenings (2.2.3.3 and 3.2.2.1.3) to create "mock infected samples". Thermal cycling conditions were as follows: Initial incubation for 2 mins at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. Fluorescence was detected at 610nm (TEX) at the end of each annealing/extension phase.

3.2.1.3.2.2 Initial duplex qPCR conditions for the M. haemomuris/GAPDH and E. coccoides/GAPDH assays

Each initial duplex qPCR consisted of 2X GoTaq (Promega), 4.5mM $MgCl_2$, forward and reverse primers (specific to either *M. haemomuris* or *E. coccoides* 16 S rRNA genes) each at 200nM and a TaqMan probe at 100nM final concentration. Primers for the host internal control (GAPDH) assay were added at 100nM each and the host internal control TaqMan probe at 50nM final concentration. Water and template DNA were added to a final volume of 25µl. Initial duplex reactions were tested with "mock infected samples" as outlined above (3.2.1.3.2.1). Each PCR was run in triplicate on the same plate. Thermal cycling was as above (3.2.1.3.2.1) and fluorescence was detected at 516nm (FAM) and 610nm (TEX) at the end of each annealing/extension phase.

3.2.1.3.2.3 Optimisation GAPDH assay qPCR primer and probe concentrations

The duplex qPCRs, specific to either *M. haemomuris* or *E. coccoides* 16S rRNA genes, were assessed for interference with the host internal control qPCR (targeting the mouse GAPDH gene) by repeating reactions with GAPDH primers/probe concentrations of 100nM/50nM, 50nM/25nM, and 12.5nM/12.5nM per reaction in a final volume of 25µl. Template DNA comprised of three different haemoplasma 16S rRNA amplicon dilutions in a background of mouse genomic DNA, as outlined above (3.2.1.3.2.1). Thermal cycling conditions and detection of fluorescence was identical to the protocol outlined above (3.2.1.3.2.2).

3.2.1.3.3 Murine haemoplasma duplex assay validation

3.2.1.3.3.1 Reaction efficiency evaluation

Reaction efficiency was assessed for the final assays by generating a standard curve, using a 10-fold dilution series of *M. haemomuris* and *E. coccoides* 16S rRNA gene amplicons in a background of mouse genomic DNA as described above (3.2.1.1.1 and 3.2.1.2.1). Dilution series were prepared immediately before use and added as 5µl template to make up 25µl duplex qPCRs comprising 200nM forward and reverse primers for either the *M. haemomuris* or *E. coccoides* assay and 100nM of their shared probe (see below), 4.5mM $MgCl_2$, 12.5nM of each internal control primer and probe. Standard curves were run in duplicate on the same qPCR plate under the conditions described in 3.2.1.3.2.2.

3.2.1.3.3.1.1 Calculating assay efficiency and slope of amplification curve

Assay efficiency and slope of the amplification curve were calculated as described for 3.2.1.1.3.3.1.

3.2.1.3.3.2 Assessment of final duplex assay cross-reactivity

Cross-reactivity of the final *M. haemomuris and E. coccoides*-specific duplex assays was assessed by challenging the *M. haemomuris*-specific assay with a single dilution of *E. coccoides* 16S rRNA gene amplicon in mouse genomic DNA (approximately 1.79E+06 copies per reaction as used for standard curve generation in 3.2.1.2.3.3). Reactions were carried out in triplicate. The equivalent experiment, using a single dilution of *M. haemomuris* 16S rRNA gene amplicon in mouse genomic DNA

(approximately 1.17E+06 copies per reaction) was carried out in triplicate for the *E. coccoides*-specific assay. Each qPCR consisted of reagents as described in 3.2.1.3.3.1 and cycling conditions and detection of fluorescence was identical to 3.2.1.3.2.2.

3.2.1.3.3.3 Assessment of final duplex assay sensitivity (limit of detection)

Template DNA amplicons were generated by conventional PCR (3.2.1.1.1 and 3.2.1.2.1).

Molecular weights of template amplicons were calculated using the Sequence Manipulation Site (303) on www.bioinformatics.org/sms2/dna_mw.html and amplicon concentrations from the conventional PCRs measured using a Qubit® dsDNA HS assay with a Qubit® 2.0 fluorophotometer (Invitrogen, UK), according to the manufacturer's instructions. The number of amplicons was calculated by dividing total DNA content by the calculated molecular weight of the amplicons.

Ten-fold serial dilutions of the amplicons generated in the conventional PCR were made in elution buffer following an initial dilution of 160,000-fold. Samples were run in triplicate using either the *M. haemomuris* or *E. coccoides* duplex qPCR assay (3.2.1.3.3.1) and thermal cycling conditions described above (3.2.1.3.2.2) using a volume of 5µl of template DNA eluate per reaction. Fluorescence was detected at 610nm (TEX) at the end of each annealing/extension phase. Limit of detection (LoD) was defined as the last dilution that yielded one or more positive qPCR results.

3.2.1.3.4 Analysis of qPCR data

Analysis was carried out as described for 3.2.1.1.4.

3.2.2 Application of the novel, species-specific duplex qPCR assays to monitor haemoplasma infection kinetics in mice

3.2.2.1 Animals

All mice were female, SPF C57BL/6 and sourced from Harlan Laboratories UK Ltd., Loughborough, UK.

3.2.2.1.1 Ethical approval

All studies involving laboratory rodents were subject to ethical review (Animal Welfare Ethical Review Body of the University of Bristol) and covered by a Home Office licence (PPL 30/2948).

3.2.2.1.2 Housing conditions

Mice were housed and monitored as previously described in 2.2.3.2.

3.2.2.1.3 Screening mice for pre-existing *M. haemomuris*-infection

One week prior to all inoculation experiments, mice were uniquely identified, allowed an acclimatisation period and had a 10µl blood sample withdrawn for generic haemoplasma qPCR analysis as outlined in 2.2.3.3.

3.2.2.1.4 Haemoplasma Inoculation

3.2.2.1.4.1 Inoculation schematic

An overview of the inoculation schematic is given in **Figure 13**.

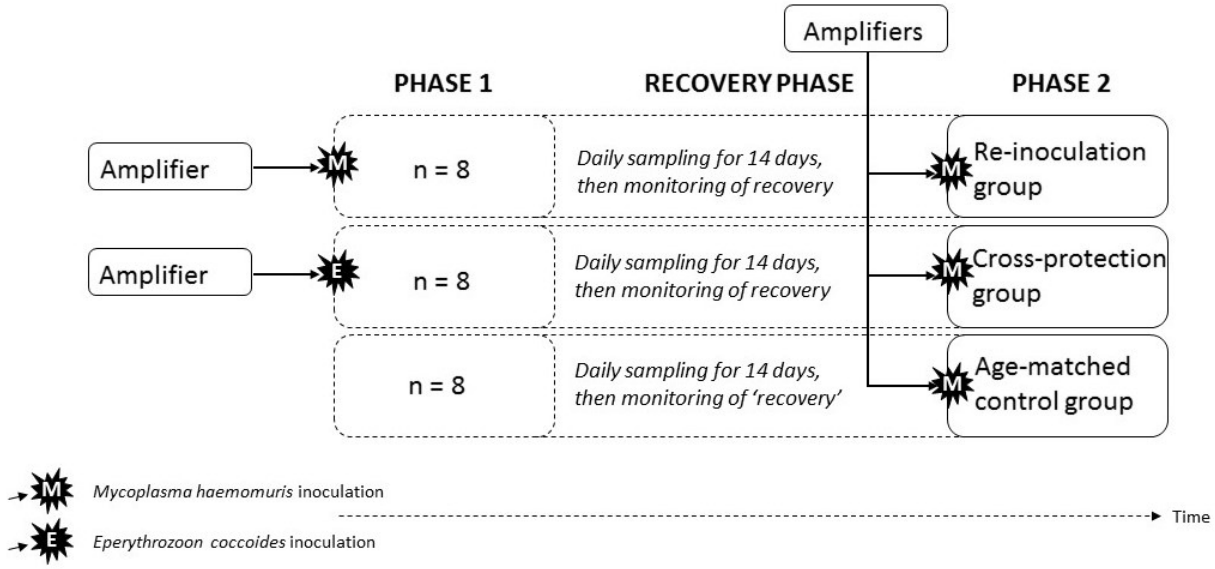


Figure 13 *Inoculation schematic to outline experimental design for the rodent haemoplasma infection kinetic studies, assessing protection from re-infection with M. haemomuris and cross-protection from M. haemomuris following E. coccoides infection. In Phase 1 of the experiment, three groups of mice were either inoculated with cryopreserved M. haemomuris (re-inoculation group), inoculated with cryopreserved E. coccoides (cross-protection group) or were not inoculated and remained naïve (age-matched control group). The mice inoculated with haemoplasmas were subsequently monitored daily by qPCR for M. haemomuris and E. coccoides genome copies per µl blood. The control group was subject to the same procedures (blood sampling) and housed under the same conditions as the groups inoculated with haemoplasmas. Following recovery from infection in the Recovery phase, all three groups were inoculated with freshly collected M. haemomuris-containing mouse blood in Phase 2 of the experiment and monitored for M. haemomuris and E. coccoides infection by qPCR.*

In brief, mice (24) were split into three groups of eight mice and each group housed in two cages of four mice each. Randomisation was achieved at the level of mouse delivery from the supplier and information regarding the original litters the mice were in was not available to the researcher. One group of eight mice was used to assess naïve infection kinetics of *M. haemomuris* and a second group was used to assess naïve infection kinetics of *E. coccoides.* Both groups were later re-inoculated with *M. haemomuris*-containing blood and an age-matched control group was inoculated at the same time with *M. haemomuris*-containing blood; this group was to control for age as well as any effects of blood sampling (including wound healing, associated stress) on immunity (see later). Phase 1 of the experiment (see **Figure 13**) represented primary infection with *M. haemomuris* of the re-inoculation group, and primary infection with *E. coccoides* of the cross-protection group. Phase 1 also included the start of experiment for the age-matched control group. The period in-between Phase 1 and Phase 2 was named the 'Recovery phase' as it represented recovery from infection with *M. haemomuris* or *E. coccoides* of the groups that had been inoculated with these haemoplasmas in Phase 1. The age-matched control group underwent identical handling with blood sampling performed during the Recovery phase, but these blood samples did not undergo further testing except for samples taken immediately prior to Phase 2 inoculations. Phase 2 of the experiment comprised re-inoculation with *M. haemomuris*-containing blood of the mice previously inoculated with *M. haemomuris* or *E. coccoides* in Phase 1. It also included inoculation with *M. haemomuris*-containing blood of the age-matched controls, representing primary infection with *M. haemomuris* of the age-matched control group.

3.2.2.1.4.2 Inoculation procedure

Inoculation procedures for all mice were identical to the procedure described in 2.2.3.4.

3.2.2.1.5 Generating inocula in amplifier mice

The experiment was split into Phase 1 and Phase 2 and each phase had to be preceded by production of amplifier mice to generate the inocula for use in the three experimental groups in both phases 1 and 2 (**Figure 13**). The amplifier mice preceding Phase 1 of the experiment were produced by the inoculation of isolates that had undergone three previous serial passages in C57BL/6 mice since sourcing the original rodent haemoplasma isolates from field mice (*Apodemus* spp.) samples. The amplifier mice preceding Phase 2 of the experiment were produced by the inoculation of isolates that had undergone four serial passages in C57BL/6 mice since sourcing the original rodent haemoplasma isolates from field mice (2.2.1).

3.2.2.1.5.1 Description of inocula used to inoculate the amplifier mice preceding Phase 1

Two nine-week-old mice were inoculated with 0.1 ml cryopreserved blood containing *M. haemomuris* or *E. coccoides*. The origin and composition of the inocula used to inoculate these laboratory mice is given in **Table 9**.

Mouse ID	Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	Haemoplasma dose in 0.1ml of inoculum per mouse
Amplifier 1	*Mycoplasma haemomuris*	Blood from a C57BL/6 mouse	20% DMSO (v/v)	1:4	1.51E+06
Amplifier 2	*Eperythrozoon coccoides*	Blood from a C57BL/6 mouse	20% DMSO (v/v)	1:4	2.55E+06

Table 9 *Origin and composition of inocula used to inoculate the amplifier mice preceding Phase 1. These mice were subsequently used to generate further inocula for Phase 1 of the experiment as described in* ***Figure 1****. Inoculum haemoplasma doses were calculated as in 2.2.1.2.2.2. DMSO, dimethyl-sulfoxide. PBS, phosphate buffered saline.*

Inocula were allowed to defrost at room temperature for 10 minutes and were inoculated intraperitoneally as previously described (2.2.3.4.1). Following inoculation, amplifier mice were monitored daily for haemoplasma infection as described previously (2.2.3.6) and exsanguinated as described above (2.2.3.7) following confirmation of infection at four dpi (infection kinetics data not shown) to produce the inocula for Phase 1. The inocula for Phase 1 underwent cryopreservation as described before (2.2.3.7) and were stored at -80°C for 13 days prior to administration to the experimental groups.

3.2.2.1.5.2 Description of inocula used to inoculate the amplifier mice preceding Phase 2

Two 9-week-old mice were inoculated with 0.1ml cryopreserved blood containing *M. haemomuris.* The origin and composition of the inocula used to inoculate these laboratory mice is given in **Table 10**.

Mouse ID	Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	Haemoplasma dose in 0.1ml of inoculum per mouse
Amplifier 3 and Amplifier 4	*Mycoplasma haemomuris*	Blood from a C57BL/6 mouse	10% Glycerol (v/v)	None	6.4E+05

Table 10 *Origin and composition of inocula used to inoculate the amplifier mice preceding Phase 2. These mice were subsequently used to generate further inocula for Phase 2 of the experiment as described in* ***Figure 1****. Haemoplasma doses were calculated as in 2.2.1.2.2.2. PBS, phosphate buffered saline.*

Following inoculation, amplifier mice were monitored for *M. haemomuris* infection as described previously (2.2.3.6) and exsanguinated as described above (2.2.3.7) on 45 dpi (infection kinetic data

not shown) to produce the inocula for Phase 2. The inocula for Phase 2 of the study were administered to the experimental groups without cryopreservation, within ten minutes of sample collection.

3.2.2.1.6 Phase 1: Primary infection kinetics of *M. haemomuris*- and *E. coccoides* in naïve mice

3.2.2.1.6.1 Re-inoculation group: Description of inoculum used for primary infection with M. haemomuris

Eight eight-week-old mice were housed in two cages of four mice each; mice '1' to '4' in cage A and mice '5' to '8' in cage B. After an acclimatisation period of one week, each mouse was inoculated with 0.1ml of a *M. haemomuris*-containing inoculum that had been prepared by defrosting 0.1ml cryopreserved blood with 0.7ml of PBS at room temperature. The inoculum used for Phase 1 of the experiment had been generated in the amplifier mice preceding Phase 1 and the composition of the cryopreserved inoculum is given in **Table 11**. Mice were inoculated in sequential order of mouse '1' to '8' using the technique described in 2.2.3.4.1.

Group inoculated	Organism	Origin	Haemoplasma copies/µl blood	Cryopreservation	Dilution ratio of inoculum in PBS	Haemoplasma dose in 0.1ml of inoculum per mouse	Number of mice inoculated
Re-inoculation group	*Mycoplasma haemomuris*	Amplifier 1	2.02E+05	20% DMSO (v/v)	1:7	2.52E+06	8
Cross-protection group	*Eperythrozoon coccoides*	Amplifier 2	3.40E+05	20% DMSO (v/v)	1:7	4.25E+06	8
Age-matched controls	None	n/a	n/a	n/a	n/a	n/a	n/a

Table 11 *Origin and composition of inocula used to inoculate the mice in Phase 1 of the experiment. Amplifier mice (**Table 9**) were killed at four days post inoculation. Inocula were cryopreserved and stored at -80 °C for 13 days until use. DMSO, dimethyl-sulfoxide. PBS, phosphate buffered saline. n/a, not applicable.*

3.2.2.1.6.2 Cross-protection group: Description of inoculum used for primary E. coccoides infection

Eight eight-week-old mice were housed as described for the re-inoculation group (3.2.2.1.6.1). After acclimatisation, each mouse was inoculated with 0.1ml of an *E. coccoides*-containing inoculum that had been prepared by defrosting 0.1ml cryopreserved blood with 0.7ml of PBS at room temperature. The order and technique of inoculation was identical to the *M. haemomuris*-inoculated group described above (3.2.2.1.6.1). The inoculum used for Phase 1 of the experiment had been generated in the amplifier mice preceding Phase 1 and the composition of the cryopreserved inoculum is given in **Table 11**.

3.2.2.1.6.3 Age-matched control group

Eight eight-week-old mice were housed as described for the re-inoculation group (3.2.2.1.6.1). No mice in this group were subject to any haemoplasma inoculation in Phase 1 of the experiment.

3.2.2.1.6.4 Monitoring of haemoplasma infection after Phase 1 inoculations

Mice in the re-inoculation group and the cross-protection group were monitored for haemoplasma infection immediately following inoculation in Phase 1 by withdrawing 10µl of blood each day on 14 consecutive days (up to 14 dpi); these blood samples underwent DNA extraction and subsequent generic haemoplasma qPCR as described in 2.2.3.6. The mice were also monitored for clinical signs of ill health at least once daily throughout the experiment.

Mice in the age-matched control group also had 10µl of blood taken on the same days (up to 14 dpi) as the re-inoculation and cross-protection groups, but the blood samples were not further analysed.

This sampling was performed to control for the possible effects of blood sampling (including wound healing, associated stress) on immunity in Phase 2 of the experiment.

3.2.2.1.7 Recovery phase in-between Phase 1 and Phase 2

After 14 dpi (3.2.2.1.6.4), in the Recovery phase, the blood sampling frequency in all of the inoculated mice in the re-inoculation group and the cross-protection group was reduced from daily to every other day, and from 31 to 61 dpi further reduced to every three days. Thereafter blood sampling frequency dropped to once a week for 13 weeks. Blood samples were subject to a generic haemoplasma qPCR assay following DNA extraction as described before 2.2.3.6. The mice were also monitored for clinical signs of ill health at least once daily throughout the experiment.

In the Recovery phase, the mice in the age-matched control group had 10µl of blood taken on the same days as the re-inoculation and cross-protection groups as described above, but the blood samples were not further analysed.

3.2.2.1.8 Phase 2: *M. haemomuris* infection kinetics in mice previously infected with *M. haemomuris* (re-inoculation group) and *E. coccoides* (cross-protection group) and primary *M. haemomuris* infection of the age-matched control group

3.2.2.1.8.1 Description of inocula used for the re-inoculation group, the cross-protection group and the age-matched control group

One-hundred and fifty-three days after primary inoculation in Phase 1, all three groups (re-inoculation group: recovered from *M. haemomuris* infection; cross-protection group: recovered from *E. coccoides* infection; age-matched control group: naive) were each inoculated with a *M. haemomuris*-containing inoculum. The inocula used for Phase 2 of the experiment had been generated in the amplifier mice preceding Phase 2 and the composition of the fresh, heparinised whole blood inocula is given in **Table 12**.

Group inoculated	Organism	Origin	Haemoplasma copies/µl blood	Cryopreservation	Dilution ratio of inoculum in PBS	Haemoplasma dose in 0.1 ml of inoculum per mouse	Number of mice inoculated
Re-inoculation group	*Mycoplasma haemomuris*	Amplifier 3	3.80E+02	None	1:1	1.90E+04	4
Cross-protection group	*Mycoplasma haemomuris*	Amplifier 4	2.40E+02	None	1:1	1.20E+04	8
Age-matched controls	*Mycoplasma haemomuris*	Amplifier 3	3.80E+02	None	1:1	1.90E+04	8

Table 12 *Origin and composition of inocula used to inoculate the mice in Phase 2 (re-inoculation with M. haemomuris of the mice previously inoculated with M. haemomuris or E. coccoides; primary M. haemomuris inoculation of the age-matched controls, see* ***Figure 13****) of the experiment. Amplifier mice (****Table 10****) were killed at 45 days post inoculation. Inocula were used fresh, without cryopreservation and at the minimal dilution to try and maximise successful inoculation of all experimental mice. PBS, phosphate buffered saline.*

Mice were inoculated in sequential order of mouse '1' to '8' in the re-inoculation group, followed by the same sequential order of the age-matched control group (using the same amplifier mouse to source inocula from; Amplifier 3, **Table 12**) and then mouse '1' to '8' in the cross-protection group (Amplifier 4, **Table 12**). The inocula (**Table 12**) were administered using the technique described in 2.2.3.4.1 within ten minutes of exsanguination of the amplifier mice (Amplifiers 3 and 4, **Table 12**).

3.2.2.1.8.2 Monitoring of haemoplasma infection after Phase 2 inoculations

Mice in the re-inoculation group, the cross-protection group and the age-matched control group were monitored for *M. haemomuris* infection immediately following inoculation in Phase 2 by

withdrawing 10µl of blood each day on 14 consecutive days; these blood samples underwent DNA extraction and subsequent *M. haemomuris*-specific qPCR as described in 3.2.1.3.3.1. The DNA extracted from the blood samples taken from the mice in the cross-protection group additionally underwent *E. coccoides*-specific qPCR as described in 3.2.1.3.3.1. to monitor for any *E. coccoides* re-emergence.

3.2.2.2 Sample collection and processing of blood samples from laboratory mice

3.2.2.2.1 Collection of inocula and post-mortem examination

At the end of the whole experiment, 14 days after the Phase 2 inoculations, all mice were exsanguinated, and samples of the blood used to generate inocula for future use, using cryopreservation as described in 2.2.3.7. A subset of the blood samples underwent centrifugation at 1000 x g for ten minutes at 4°C to derive plasma samples for storage for use in potential future serological studies; these samples were stored at -80°C. Post-mortem examinations were carried out on all mice and the spleens were weighed, with splenomegaly defined as a splenic weight of greater than 0.4% bodyweight (276) as no healthy control mice from the same population were available for comparison of splenic weight.

3.2.2.2.2 Collection of blood samples for qPCR and development of the haemoplasma species-specific qPCR assays

Unless otherwise indicated, the method used for blood sampling for qPCR was identical to the procedure described in 2.2.3.3.2.1. Samples were stored at -20°C until DNA extraction and further use.

3.2.2.2.3 DNA extraction for qPCR and development of the haemoplasma species-specific qPCR assays

DNA was extracted manually using single-spin column silica membrane technology as previously described (2.2.1.2.1) up to and including 14 dpi of Phase 1. After 14 dpi (3.2.2.1.6) DNA extraction was streamlined to increase efficiency by replacing the single-spin columns with 8-well-strips (NucleoSpin® 8 Blood, Macherey-Nagel, UK). In brief, samples were defrosted, diluted with PBS to a final volume of 200µl and mixed with 25 µl of Proteinase K and 200µl of lysis buffer as described for the single-spin columns. This was followed by incubation for 30 minutes on a rocking platform. Ethanol was added to the mixture to a final volume of 410µl and the preparation loaded on the silica membrane strips, as for single-spin columns. The strips were mounted onto a 96-well rack/collection block, allowing for simultaneous processing of up to four 8-well-strips per block. After an initial spin at 1,600 x g for two minutes, the flow-through was discarded and washing steps were carried out as for the single-spin columns (2x 500µl of wash buffer 1, 'BW') but speed was adjusted to 3,000 x g for ten minutes. After the final wash (wash buffer 2, 'B5') and spinning at 3,000 x g for 10 minutes, 50µl of elution buffer ('BE') was added and incubated for ten minutes at room temperature. This was followed by spinning at 1,600 x g for two minutes into clean collection tubes. The flow-through was kept for subsequent qPCR and stored at -80°C until further use.

3.2.2.3 Analysis of mouse blood samples for haemoplasma genome copies

3.2.2.3.1 Conditions of the generic haemoplasma qPCR assay

Samples from Phase 1 of the study (3.2.2.1.6) were analysed for haemoplasma genome copies per µl blood using the generic haemoplasma qPCR assay (HF-group) described previously (2.2.1.2.2). Negative (water) and positive controls of known *M. haemofelis* genome copy number and a separately run host control qPCR were used and were identical to the ones described for previous

studies (2.2.3.3.3). All qPCRs were performed on the same Mx3005P qPCR thermal cycling system (Agilent, UK).

3.2.2.3.1.1 Analysis of qPCR data

Analysis of data from the generic haemoplasma qPCR assay was carried out as described for 2.2.1.2.2.2. Normalisation of Ct values and calculation of relative genome copies per µl blood was performed as described in 2.2.1.2.2.2.1 and 2.2.1.2.2.2.2.

3.2.2.3.2 Conditions of the haemoplasma species-specific qPCR assays

Samples from Phase 2 of the study (3.2.2.1.8) were analysed for haemoplasma genome copies per µl blood using the species-specific *M. haemomuris* and *E. coccoides* duplex qPCR assays described in 3.2.1.3.3.1. Thermal cycling conditions were identical to 3.2.1.3.2.2. Positive control samples of known *M. haemomuris* and *E. coccoides* genome copy number and negative control samples (water) were included on each plate. All qPCRs were performed on the same Mx3005P qPCR thermal cycling system (Agilent, UK).

3.2.2.3.2.1 Analysis of qPCR data

Analysis of data from the haemoplasma species-specific qPCR assays was carried out as described for 3.2.1.1.4. Normalisation of Ct values and calculation of relative genome copies per µl blood was performed as described in 2.2.1.2.2.2.1 and 2.2.1.2.2.2.2, using assay efficiencies of 0.980 for the *M. haemomuris*-specific qPCR assay and 0.998 for the *E. coccoides*-specific assay.

3.2.2.4 Statistical analyses

3.2.2.4.1 Power calculations

No pilot data or previous research on protective immunity following *M. haemomuris* and *E. coccoides* infection were available to guide power calculations. Based on available data on feline haemoplasma infection with *M. haemofelis* (107, 295) or '*Ca.* M. turicensis' (124), the required group sizes to detect significant differences in *M. haemomuris* infection kinetics were estimated using G*Power v3.1.9.2 (304). In brief, an *a priori* analysis was run on the difference between two independent means with two equal group sizes. Significance was defined as $p<0.05$ and statistical power set to 0.8. Based on full protection for feline *M. haemofelis* infection, effect size was estimated at 2.0 (meaning the difference between the group means had to be greater than two common standard deviations) using published *M. haemofelis* data at peak parasitaemia and the effect size calculator tool cNORM (305) and using the effect size classification by Cohen (306) and Hattie and Timperley (307). Required group sizes were calculated as six individuals per group and arbitrarily increased by two mice per group to account for unknown infection kinetics and a larger standard deviation due to smaller blood sampling volumes collected from mice compared to cats.

3.2.2.4.2 Calculation of doubling time

Doubling time (Dt) over the observed period of exponential growth was calculated using the following formula:

$$Dt = \frac{\text{duration} * \log(2)}{\log(\text{end haemoplasma genome copies/µl})/\log(\text{initial haemoplasma genome copies/µl})}$$

where Dt = doubling time and log = logarithm to the base of 2

3.2.2.4.3 Statistical assessment of protective immunity

Area-under-the-curve (AUC) values were calculated for individual mice. Extraction failures or missed days (due to clinical concerns, n=1) were replaced by averages of preceding/following day for the

individual mouse. Due to the very small sample size (n=4 to n=8), and associated predicted lack of normal distribution, the data were treated as non-parametric in nature. The statistical software package SPSS v. 24 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) was used to calculate Mann-Whitney-U statistics to compare groups. Significance was defined as $p<0.05$.

3.3 RESULTS

3.3.1 Haemoplasma species-specific duplex qPCR assays for two novel rodent haemoplasma isolates of *M. haemomuris* and *E. coccoides*

3.3.1.1 Primers and probe for the M. haemomuris singleplex qPCR assay

Six primer pairs and respective TaqMan probes designed to binding sites on the *M. haemomuris* 16S rRNA gene were selected for maximal sequence divergence to the *E. coccoides* 16S rRNA gene, and are shown in **Table 13**.

Assay name	Forward Primer 5'-3'	Probe 5'-3'	Reverse Primer 3'-5'
Mhm-1	GCGAAAGCCTGATGGAGTG	AAAGCGAGCGCAGGCGGATT	CGTTGAGCGGCTGCATT
Mhm-2	GCGCAAGCCACTTGGAG	CCACAATGGGCGAAAGCCTGA	TACCATCATCATTTTCCCCAAT
Mhm-3	AGCCACTTGGAGATTGGAGTATT	CCACAATGGGCGAAAGCCTGA	TACCATCATCATTTTCCCCAAT
Mhm-4	GCGAAAGCCTGATGGAGT	TGCCATGTGAACGATGAAGGTCTT	TACCATCATCATTTTCCCCAAT
Mhm-5	AGCCACTTGGAGATTGGAGTATT	AAAGCGAGCGCAGGCGGATT	CGTTGAGCGGCTGCATT
Mhm-6	TGTATGCGCAGAATACTGTTTTTC	AACACCAGAGGCGAAGGCGAAA	GAGACTCAAGTCCCAACATCTAATATC

Table 13 *Primer and probe (oligonucleotide) sequences designed for species-specific quantitative detection of the M. haemomuris 16S rRNA gene.*

3.3.1.2 Results for initial M. haemomuris singleplex qPCR assays and cross-reactivity using SYBR Green 1

The six primer pairs selected for *M. haemomuris*-specific qPCR detection (3.3.1.1) were subsequently assessed for reaction specificity at the primer level in a singleplex SYBR Green 1 qPCR. All samples, known to contain *M. haemomuris* DNA (3.2.1.1.3.1) gave positive results (**Table 14**).

Assay name	Ct results for *M. haemomuris* positive sample		Ct results for mouse DNA positive sample	Ct result for negative (water) samples	
Mhm-1	25.58	25.78	33.08	35.10	34.60
Mhm-2	27.12	26.66	No Ct	No Ct	No Ct
Mhm-3	25.56	25.84	33.06	33.11	34.61
Mhm-4	26.28	25.79	33.28	No Ct	No Ct
Mhm-5	28.32	28.50	28.42	28.86	28.59
Mhm-6	25.92	26.52	No Ct	No Ct	No Ct

Table 14 *Threshold cycle results (Ct) for the initial M. haemomuris singleplex qPCR assays using SYBR Green 1 as a reporter. All M. haemomuris-positive samples contained the same amount of M. haemomuris target DNA (same dilution of PCR amplicon).*

The peak dissociation temperatures of the products produced by primer pairs of assays Mhm-1, Mhm-2, Mhm-3 and Mhm-6 were approximately 86°C, suggesting similar PCR products. Primer pairs from assays Mhm-4 and Mhm-5 produced different peak dissociation temperatures of approximately 77°C and 80°C respectively, suggesting different products, possibly as a result of low specificity (Mhm-4, as this product also appeared with these primers when mouse DNA was used as template) or primer-dimer formation (Mhm-5, as the dissociation temperature was lower and was possibly at the primer-dimer dissociation temperature). The assays Mhm-2 and Mhm-6 resulted in no detected Ct value with *E. coccoides* genomic DNA as template (3.2.1.1.3.2), indicating a lack of cross-reactivity. Primer pairs from assays Mhm-2 and Mhm-6 were chosen for further evaluation in view of their apparent specificity to the target haemoplasma species.

3.3.1.3 Reaction efficiencies of the M. haemomuris singleplex qPCR assay using SYBR Green 1

Ten-fold dilutions of *M. haemomuris* target DNA (the 16S rRNA gene amplicons generated in 3.2.1.1.1) were used to generate a standard curve for assays Mhm-2 and Mhm-6 using SYBR Green 1

as a reporter fluorophore. For assay Mhm-2, 1.0E+04-fold dilutions produced a correlation coefficient of 0.999, the slope of the standard curve was -3.523 and qPCR efficiency 92.2% (**Figure 14**). For assay Mhm-6, 1.0E+04-fold dilutions produced a correlation coefficient of 0.997, the slope of the standard curve was -3.355 and qPCR efficiency 98.6% (**Figure 15**).

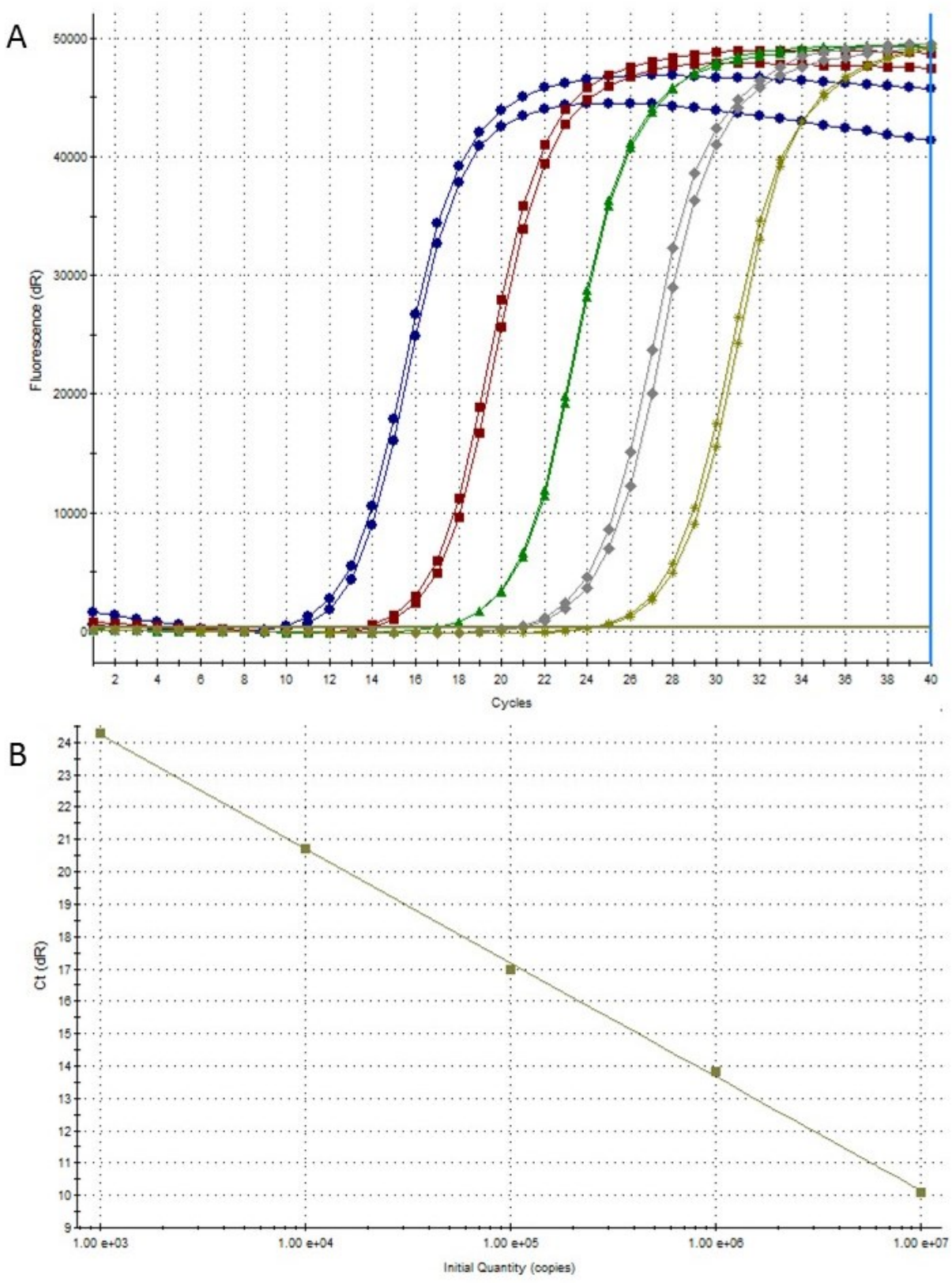


Figure 14 *Amplification plot (Panel A) and standard curve (Panel B) for M. haemomuris singleplex qPCR assay Mhm-2 using SYBR Green 1.*

Panel A: *Cycle numbers are plotted on the x-axis and relative fluorescence units are plotted on the y-axis with threshold fluorescence indicated by the green horizontal line and 40 cycle cut-off by the blue vertical line.* ***Panel B:*** *Standard curve as generated from duplicate dilutions over 10E+04-fold. Dilution from the starting quantity is plotted on the x-axis and threshold cycles (Ct) are plotted on the y-axis.*

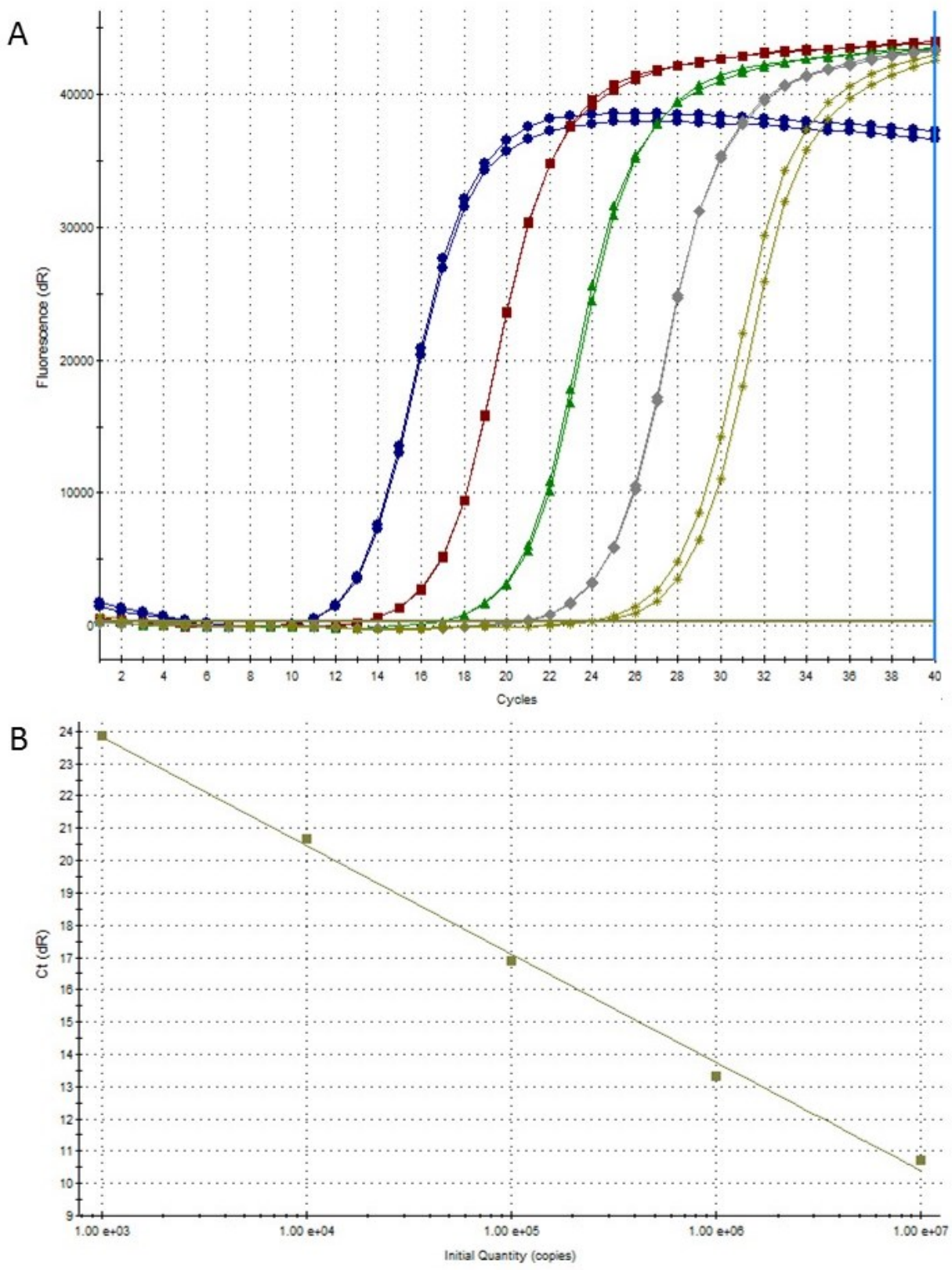


Figure 15 *Amplification plot (Panel A) and standard curve (Panel B) for M. haemomuris singleplex qPCR assay Mhm-6 using SYBR Green 1.*
Panel A: *Cycle numbers are plotted on the x-axis and relative fluorescence units are plotted on the y-axis with threshold fluorescence indicated by the green horizontal line and 40 cycle cut-off by the blue vertical line.* ***Panel B:*** *Standard curve as generated from duplicate dilutions over 10E+04-fold. Dilution from the starting quantity is plotted on the x-axis and threshold cycles (Ct) are plotted on the y-axis.*

Assay Mhm-6 was subsequently selected to be converted into a *M. haemomuris*-specific qPCR assay using a TaqMan probe, and duplexing with a mouse GAPDH internal control qPCR assay to confirm successful DNA extraction and lack of PCR inhibitors in the sample.

3.3.1.4 Development of a M. haemomuris/mouse GAPDH duplex qPCR assay

3.3.1.4.1 Performance and optimisation of the *M. haemomuris TaqMan qPCR* singleplex assay in mouse DNA background

Under initial singleplex conditions, using a TEX-labelled TaqMan probe (3.2.1.3.2.1), the *M. haemomuris* assay Mhm-6 produced consistent Ct values when used with three different concentrations of *M. haemomuris* template DNA (16S rRNA gene amplicon dilutions) in a mouse DNA background (**Table 15**).

M. haemomuris DNA template concentration	Ct of singleplex assay, using a TEX-labelled TaqMan probe		
'high'	20.78	20.79	20.76
'medium'	24.34	24.23	24.25
'low'	28.18	28.01	28.26

Table 15 *Threshold cycle (Ct) results of the M. haemomuris singleplex qPCR assay Mhm-6 using a TEX-labelled TaqMan probe as a reporter. Mycoplasma haemomuris target DNA (M. haemomuris 16S rRNA gene amplicons) was evaluated at three different concentrations of M. haemomuris template DNA in a mouse DNA background.*

It was determined that the probe concentration could be lowered to 100nM per reaction without sacrificing adversely qPCR performance, using a threshold of 300 relative fluorescence units (**Table 16**).

Probe concentration (nM)	Ct of singleplex assay, using a TEX-labelled TaqMan probe		
200	18.42	18.65	18.64
100	19.27	19.08	19.04
50	19.99	19.86	19.89

Table 16 *Threshold cycle (Ct) results of the M. haemomuris* singleplex *qPCR assay Mhm-6 using a TEX-labelled TaqMan probe as a reporter. The probe was used at three different concentrations. Mycoplasma haemomuris target DNA (M. haemomuris 16S rRNA gene) was used at the same concentration in a mouse DNA background.*

3.3.1.4.2 Optimising the *M. haemomuris* TaqMan duplex qPCR assay in mouse DNA background

Under initial duplex conditions (3.2.1.3.2.2), addition of the GAPDH qPCR assay resulted in different Ct values for the Mhm-6 assay compared to singleplex Ct values at all three *M. haemomuris* template DNA concentrations (**Table 17**).

M. haemomuris DNA template	Ct of singleplex assay (range, see **Table 15**)	Ct of *M. haemomuris/GAPDH* duplex assay: GAPDH1 concentration*			Ct of *M. haemomuris/GAPDH* duplex assay: GAPDH2 concentration*			Ct of *M. haemomuris/GAPDH* duplex assay: GAPDH3 concentration*		
'high'	20.76 - 20.79	21.77	21.63	21.63	20.99	20.65	20.67	21.06	21.00	20.79
'medium'	24.23 - 24.34	25.85	25.64	25.66	24.45	24.45	24.47	24.19	24.14	24.51
'low'	28.01 - 28.26	31.95	31.92	32.19	29.68	29.87	29.81	28.57	28.38	28.17

Table 17 *Threshold cycle (Ct) results of the M. haemomuris-specific duplex qPCR assay Mhm-6 using a TEX-labelled TaqMan probe as a reporter. Mycoplasma haemomuris template DNA (M. haemomuris 16S rRNA gene amplicons) was evaluated at three different concentrations in a mouse DNA background.*
**GAPDH1, primers and probe for GAPDH assay added at a final concentration of 100nM/50nM respectively; GAPDH2, primers and probe for GAPDH assay added at a final concentration of 50nM/25nM respectively; GAPDH3, primers and probe for GAPDH assay added at a final concentration of 12.5nM/12.5nM respectively; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.*

Of the different combinations of GAPDH primer and probe concentrations, 12.5nM of each primer and 12.5nM of probe was found not to compete with the *M. haemomuris* qPCR assay at all three *M.*

haemomuris template DNA concentrations. Mouse DNA remained detectable with a Ct range of 18.67 to 19.91 on the mouse GAPDH qPCR assay.

3.3.1.4.3 Reaction efficiency of the final *M. haemomuris* duplex qPCR assay

Ten-fold dilutions of *M. haemomuris* template DNA (16S rRNA gene amplicons, 3.2.1.1.1) in mouse genomic DNA background were used to generate a standard curve for assay Mhm-6 using a TEX-labelled TaqMan probe as a reporter for the *M. haemomuris* 16S rRNA gene and a FAM-labelled TaqMan probe as a reporter for the mouse internal control GAPDH gene assay. Final primers and probes and their respective concentrations following optimisation are shown in **Table 18**.

Assay	Forward Primer 5'-3'	Sense Taqman Probe 5'-3'	Reverse Primer 5'-3'
M. haemomuris-specific assay (Mhm-6)	TGT**A**TGC**G**C**A**GAATACT**G**TT**TT**TC	TEX-AACACCAGAGGCGAAGGCGAAA-BHQ2	G**AGA**CTC**AA**G**T**CCC**AA**CA**T**CTAATA**T**C
Final oligonucleotide concentration (in 25µl)	200nM	100nM	200nM
Host internal control assay (feline/mouse GAPDH)	GCTGCCCAGAACATCATCC	FAM-TCACTGGCATGGCCTTCCGT-BHQ1	CACAGGCAGCACCTAGACTG
Final oligonucleotide concentration (in 25 µl)	12.5nM	12.5nM	12.5nM

Table 18 *Primers and probes (oligonucleotide) sequences used for quantitative detection of the M. haemomuris 16S rRNA gene and of host genomic DNA for the GAPDH gene. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxyfluorescein; TEX, Texas Red; BHQ1, Black Hole Quencher 1; BHQ2, Black Hole Quencher 2. Nucleotide-sequence mismatches to E. coccoides are shown in bold and shaded text.*

Over 1.0E+06-fold dilutions the correlation coefficient was 0.999, the slope of the standard curve was -3.370 and qPCR efficiency 98.0% (**Figure 16**).

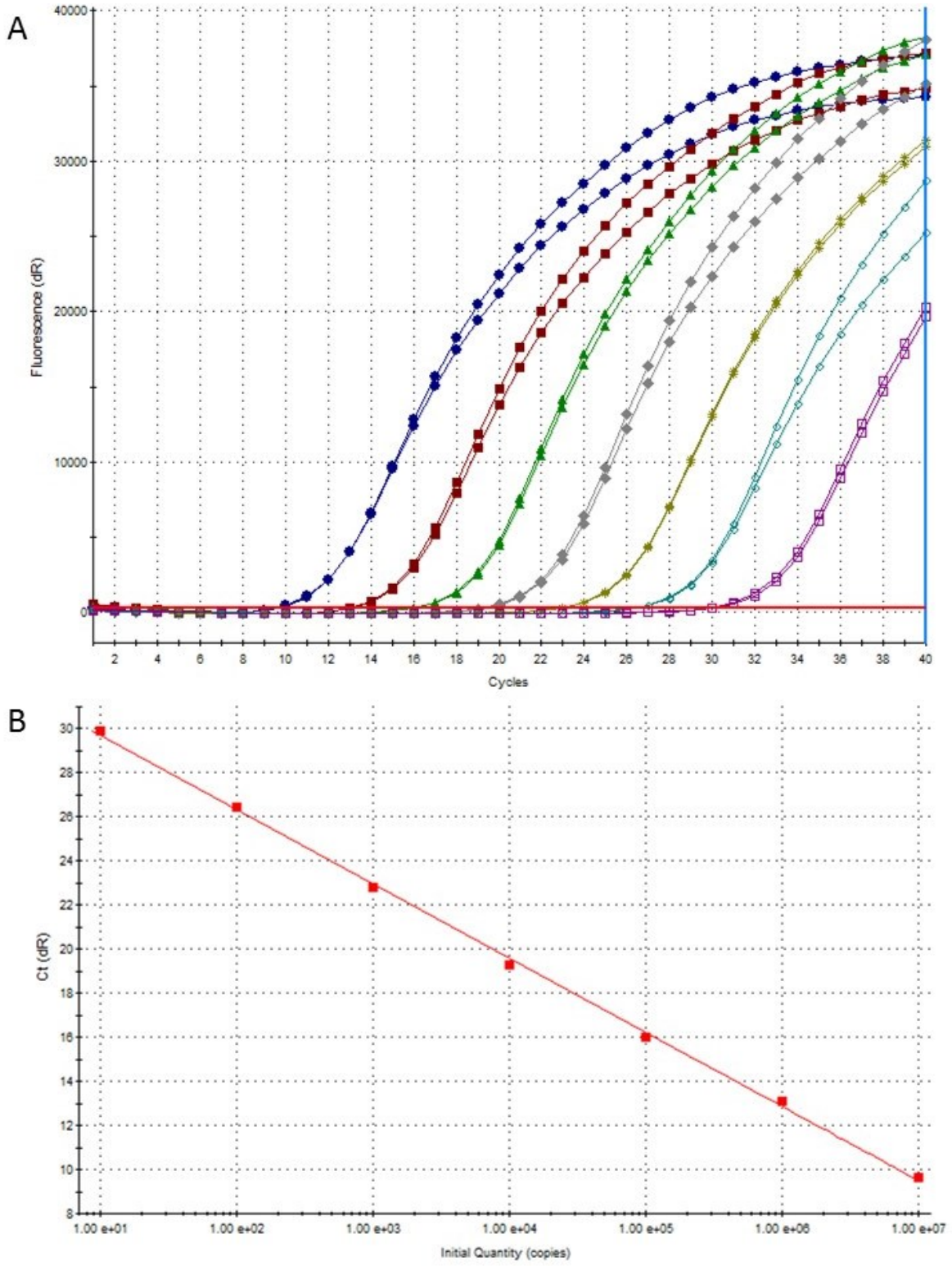


Figure 16 *Amplification plot (Panel A) and standard curve (Panel B) for the M. haemomuris singleplex qPCR assay Mhm-6 using a TEX-labelled TaqMan probe.*
Panel A: *Cycle numbers are plotted on the x-axis and relative fluorescence units are plotted on the y-axis with threshold fluorescence indicated by the red horizontal line and 40 cycle cut-off by the blue vertical line.* ***Panel B:*** *Standard curve as generated from duplicate dilutions over 1.0E+06-fold. Dilution from starting quantity is plotted on the x-axis and threshold cycles (Ct) are plotted on the y-axis.*

3.3.1.5 Primers and probe for the E. coccoides singleplex qPCR assay

Four primer pairs and respective TaqMan probes designed to binding sites on the *E. coccoides* 16S rRNA gene were selected for sequence divergence to the *M. haemomuris* 16S rRNA gene are shown in **Table 19**.

Assay name	Forward Primer 5'-3'	Probe 5'-3'	Reverse Primer 3'-5'
Ecoc-7	GACGAAAGTCTGATGGAGCAA	AAAGCAAGCGCAGGCGGATG	ACTTCCTACCACATTCTAGACGAATA
Ecoc-8	ACAGTTGTTTGCACCGAATACTA	AACACCAGAGGCGAAGGCGAAA*	ACAGCACCGAAGCTCTAACC
Ecoc-9	AATACTATTCGTCTAGAATGTGGTAGG	GAACACCAGAGGCGAAGGCGA	ACAGCACCGAAGCTCTAACC
Ecoc-10	TGTGGTAGGAAGTTTTGGAATTAAATA	AACACCAGAGGCGAAGGCGAAA*	ACAGCACCGAAGCTCTAACC

Table 19 *Primer and probe (oligonucleotide) sequences designed for species-specific quantitative detection of the E. coccoides 16S rRNA gene. Asterisks (*) indicate an identical probe to assay Mhm-6 (**Table 13**).*

3.3.1.6 Results for initial E. coccoides singleplex qPCR assays and cross-reactivity using SYBR Geen 1

The six primer pairs selected for *E. coccoides*-specific qPCR detection (3.3.1.5) were subsequently assessed for reaction specificity at the primer level in a singleplex SYBR Green 1 qPCR. All samples known to contain *E. coccoides* DNA (3.2.1.2.3.1) gave positive results (**Table 20**).

Assay name	Ct results for *E. coccoides DNA positive sample*		Ct results for mouse DNA positive sample	Ct result for negative (water) samples	
Ecoc-7	25.94	25.63	No Ct	No Ct	No Ct
Ecoc-8	24.74	25.23	No Ct	No Ct	No Ct
Ecoc-9	24.42	24.81	32.4	32.4	39.56
Ecoc-10	25.52	25.41	No Ct	No Ct	No Ct

Table 20 *Threshold cycle results (Ct) for the initial E. coccoides singleplex qPCR assays using SYBR Green 1 as a reporter. All E. coccoides-positive samples were known to contain the same amount of E. coccoides target DNA.*

The peak dissociation temperatures of the products produced by the different primer pairs of the Ecoc-7, Ecoc-8 and Ecoc-10 assays were approximately 85°C, suggesting that similar PCR products had been amplified. Primer pairs from assay Ecoc-9 produced a different product (peak dissociation temperature approximately 82°C) with mouse DNA and no template (water), suggesting primer-dimer formation. Assay Ecoc-9 was hence excluded from further evaluation. Amplification with the primer pairs from assays Ecoc-7, Ecoc-8 and Ecoc-10 with *M. haemomuris* genomic DNA (3.2.1.2.3.2) showed no cross-reactivity. Two primer pairs from assays Ecoc-8 and Ecoc-10 were chosen for further evaluation to develop an *E. coccoides*-specific qPCR assay (**Table 20**).

3.3.1.7 Reaction efficiencies of the E. coccoides singleplex assay using SYBR green

Ten-fold dilutions of *E. coccoides* template DNA (16S rRNA gene amplicons, 3.2.1.2.1) were used to generate a standard curve for assays Ecoc-8 and Ecoc-10 using SYBR Green 1 as a reporter fluorophore. For assay Ecoc-8, 1.0E+04-fold dilutions produced a correlation coefficient of 0.999, the slope of the standard curve was -3.608 and qPCR efficiency 89.3% (**Figure 17**). For assay Ecoc-10, 1.0E+04-fold dilutions produced a correlation coefficient of 1.000, the slope of the standard curve was -3.638 and qPCR efficiency 88.8% (**Figure 18**).

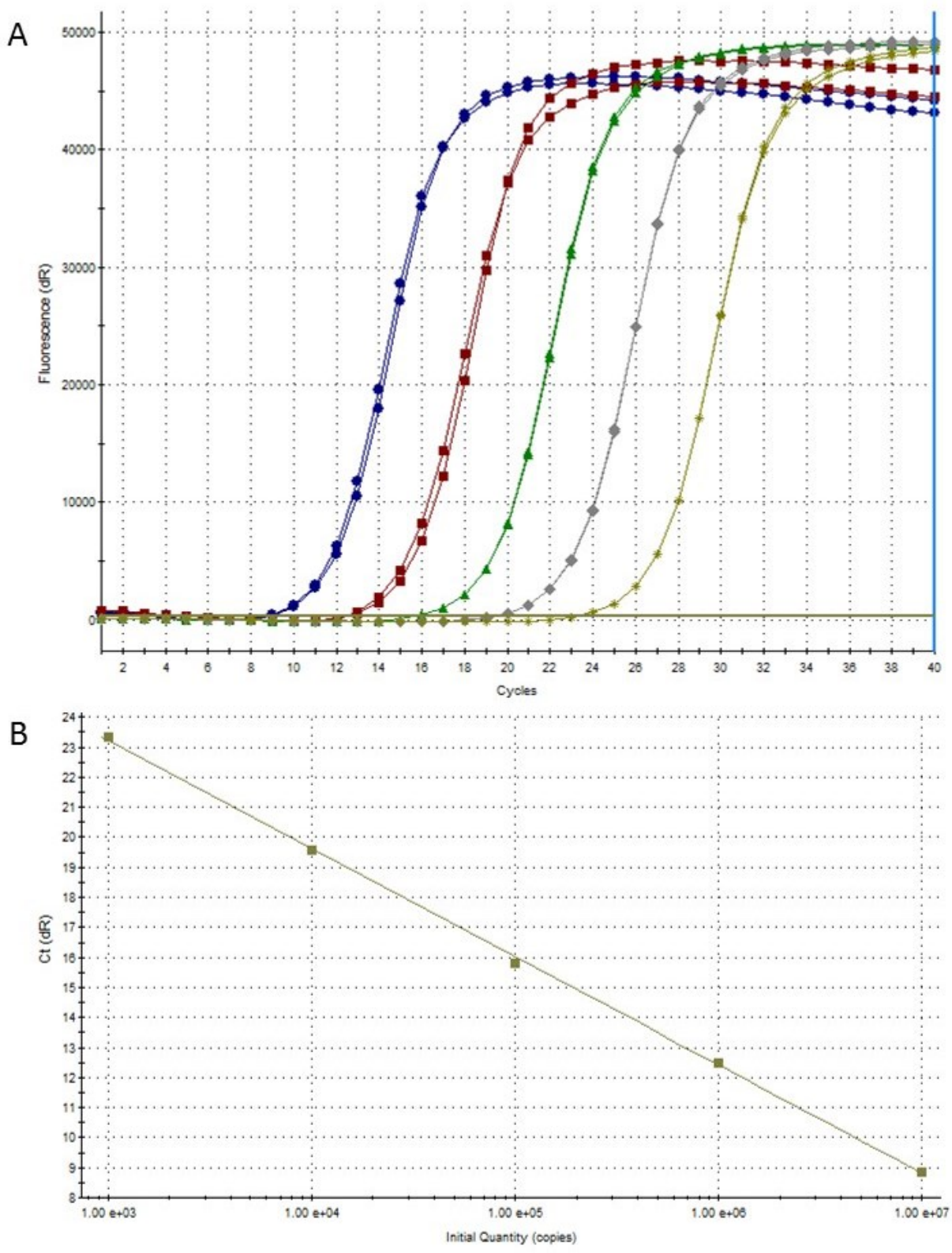


Figure 17 *Amplification plot (Panel A) and standard curve (Panel B) for the Ecoc-8 singleplex qPCR assay using SYBR Green 1.*

Panel A: *Cycle numbers are plotted on the x-axis and relative fluorescence units are plotted on the y-axis with threshold fluorescence indicated by the green horizontal line and 40 cycle cut-off by the blue vertical line.* ***Panel B:*** *Standard curve as generated from duplicate dilutions over 10E+04-fold. Dilution from starting quantity is plotted on the x-axis and threshold cycles (Ct) are plotted on the y-axis.*

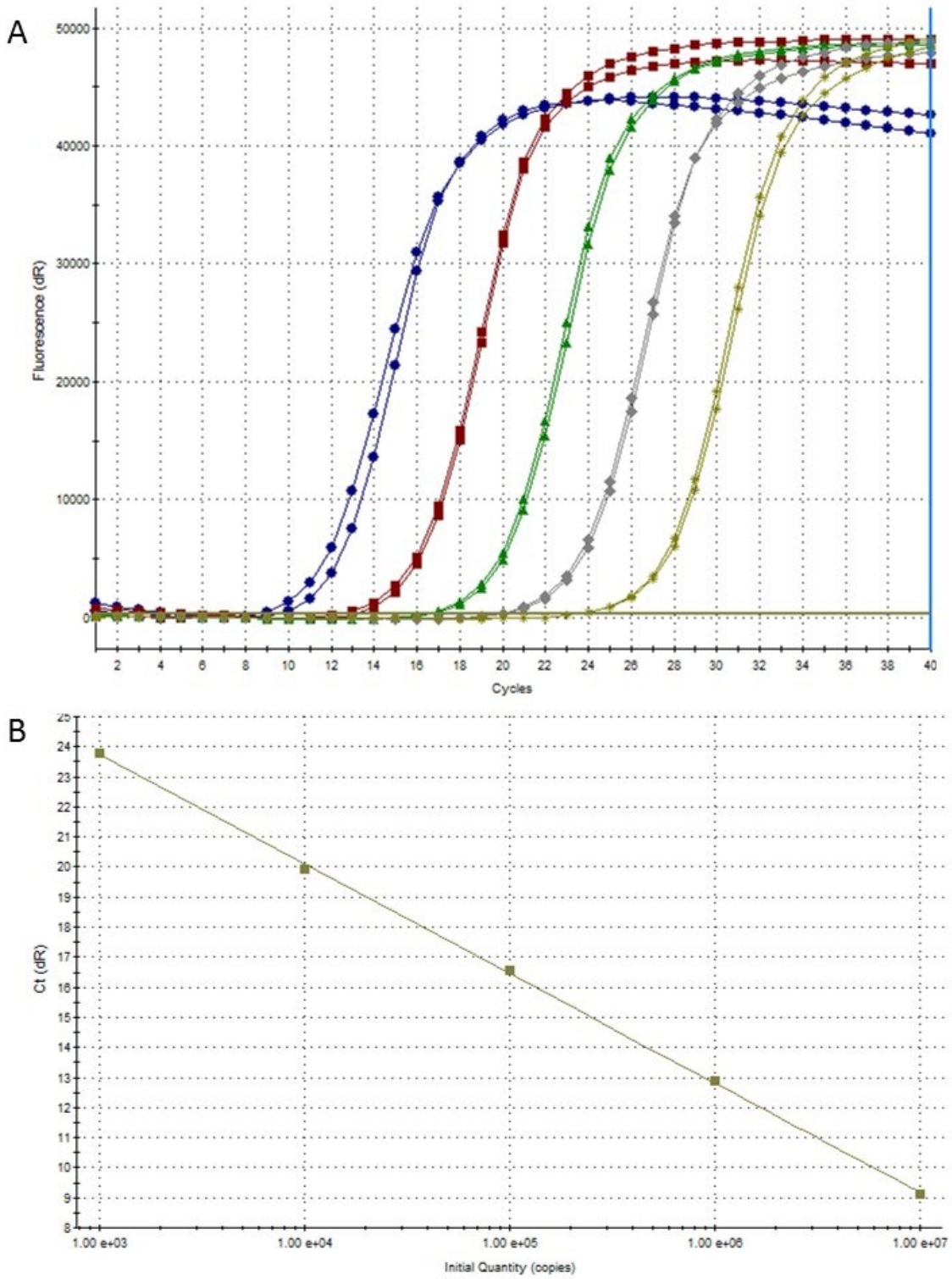


Figure 18 *Amplification plot (Panel A) and standard curve (Panel B) for the Ecoc-10 singleplex qPCR assay using SYBR Green 1.*

Panel A: *Cycle numbers are plotted on the x-axis and relative fluorescence units are plotted on the y-axis with threshold fluorescence indicated by the green horizontal line and 40 cycle cut-off by the blue vertical line.* ***Panel B:*** *Standard curve as generated from duplicate dilutions over 10E+04-fold. Dilution from the starting quantity is plotted on the x-axis and threshold cycles (Ct) are plotted on the y-axis.*

Assay Ecoc-8 was subsequently selected to be converted into a *E. coccoides*-specific qPCR assay using a TaqMan probe, and duplexing with a mouse internal control GAPDH internal control qPCR assay to confirm successful DNA extraction and lack of PCR inhibitors in the sample.

3.3.1.8 Development of a. E. coccoides/mouse GAPDH duplex qPCR assay

3.3.1.8.1 Performance and optimisation of the *E. coccoides TaqMan* singleplex qPCR assay in mouse DNA background

Under initial singleplex conditions, using a TEX-labelled TaqMan probe (3.2.1.3.2.1) the *E. coccoides*-specific qPCR assay Ecoc-8 produced consistent Ct values when used on different *E. coccoides* target amplicon dilutions in a mouse DNA background (**Table 21**).

E. coccoides DNA template concentration	Ct of singleplex assay, using a TEX-labelled TaqMan probe		
'high'	20.39	20.55	20.64
'medium'	24.07	23.96	24.23
'low'	27.38	28.13	27.84

Table 21 *Threshold cycle (Ct) results of the E. coccoides-specific singleplex qPCR assay Ecoc-8 using a TEX-labelled TaqMan probe to evaluate three different concentrations of E. coccoides template DNA amplicons in a mouse DNA background.*

It was determined that the probe concentration could be lowered to 100nM per reaction without sacrificing qPCR performance using a threshold of 300 relative fluorescence units (**Table 22**).

Probe concentration (nM)	Ct of singleplex assay, using a TEX-labelled TaqMan probe		
200	18.16	18.07	18.09
100	18.89	18.67	18.69
50	19.49	19.69	19.33

Table 22 *Threshold cycle (Ct) results of the E. coccoides-specific* singleplex *qPCR assay Ecoc-8 using a TEX-labelled TaqMan probe as a reporter. The probe was used at three different concentrations. Eperythrozoon coccoides target DNA (E. coccoides 16S rRNA gene) was used at the same concentrations in a mouse DNA background.*

3.3.1.8.2 Optimising the *E. coccoides* duplex assay in mouse DNA background

Under initial duplex conditions (3.2.1.3.2.2), addition of the GAPDH qPCR assay resulted in different Ct values for the Ecoc-8 assay compared to singleplex Ct values at medium and low *E. coccoides* template DNA concentrations (**Table 23**).

E. coccoides DNA template	Ct of singleplex assay (range, see **Table 21**)	Ct of *M. haemomuris/GAPDH* duplex assay: GAPDH1 concentration*			Ct of *M. haemomuris/GAPDH* duplex assay: GAPDH2 concentration*			Ct of *M. haemomuris/GAPDH* duplex assay: GAPDH3 concentration*		
'high'	20.39 - 20.64	20.68	20.83	20.71	20.63	20.72	20.80	20.64	20.35	20.50
'medium'	23.96 - 24.23	24.50	24.77	24.94	24.39	24.17	24.17	23.98	24.05	23.79
'low'	27.38 - 27.84	No Ct	No Ct	No Ct	28.61	28.43	28.23	27.67	27.66	27.24

Table 23 *Threshold cycle (Ct) results of the E. coccoides-specific duplex qPCR assay Ecoc-8 using a TEX-labelled TaqMan probe as a reporter. Eperythrozoon coccoides template DNA (E. coccoides 16S rRNA gene amplicons) was evaluated at three different concentrations in a mouse DNA background.*
GAPDH1, primers and probe for GAPDH assay added at a final concentration of 100nM/50nM respectively; GAPDH2, primers and probe for GAPDH assay added at a final concentration of 50nM/25nM respectively; GAPDH3, primers and probe for GAPDH assay added at a final concentration of 12.5nM/12.5nM respectively; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase.

Of the different combinations of GAPDH primer and probe concentrations, 12.5nM of each primer and 12.5nM of probe was found not to compete with the *E. coccoides*-specific qPCR assay at all three *M. haemomuris* template concentrations. Mouse DNA remained detectable with a Ct range of range 17.86 to 18.97 on the mouse GAPDH qPCR assay.

3.3.1.8.3 Reaction efficiency of the final *E. coccoides* duplex qPCR assay

Ten-fold dilutions of *E. coccoides* template DNA (16S rRNA gene amplicons, 3.2.1.2.1) in mouse genomic DNA background were used to generate a standard curve for qPCR assay Ecoc-8 using a TEX-labelled TaqMan probe as a reporter for the *E. coccoides* 16S rRNA gene and a FAM-labelled

TaqMan probe as a reporter for the mouse internal control GAPDH gene assay. Final primers and probes and their respective concentrations following optimisation are shown in **Table 24**. The probe used for the Ecoc-8 assay was identical to the probe used for the Mhm-6 assay (**Table 18**).

Assay	Forward Primer 5'-3'	Sense Taqman Probe 5'-3'	Reverse Primer 5'-3'
E. coccoides-specific assay *(Ecoc-8*)	AC**A**GTTGT**T**TGC**A**C**C**GAATACT**A**	TEX-AACACCAGAGGCGAAGGCGAAA-BHQ2	ACA**G**C**A**C**C**GA**AG**CTCT**A**T**AC**C
Final oligonucleotide concentration (in 25 µl)	200nM	100nM	200nM
Host internal control (feline/mouse GAPDH)	GCTGCCCAGAACATCATCC	FAM-TCACTGGCATGGCCTTCCGT-BHQ1	CACAGGCAGCACCTAGACTG
Final oligonucleotide concentration (in 25 µl)	12.5nM	12.5nM	12.5nM

Table 24 *Primers and probes (oligonucleotide) sequences used for quantitative detection of the M. haemomuris 16S rRNA gene and of host genomic DNA for the GAPDH gene. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxyfluorescein; TEX, Texas Red; BHQ, Black Hole Quencher; BHQ2, Black Hole Quencher 2. Nucleotide-sequence mismatches to the non-target species (E. coccoides) are shown in bold and shaded text.*

For assay Ecoc-8, over 1.0E+06-fold dilutions of *E. coccoides* template DNA produced a correlation coefficient of 0.999, the slope of the standard curve was -3.332 and qPCR efficiency 99.6% (**Figure 19**).

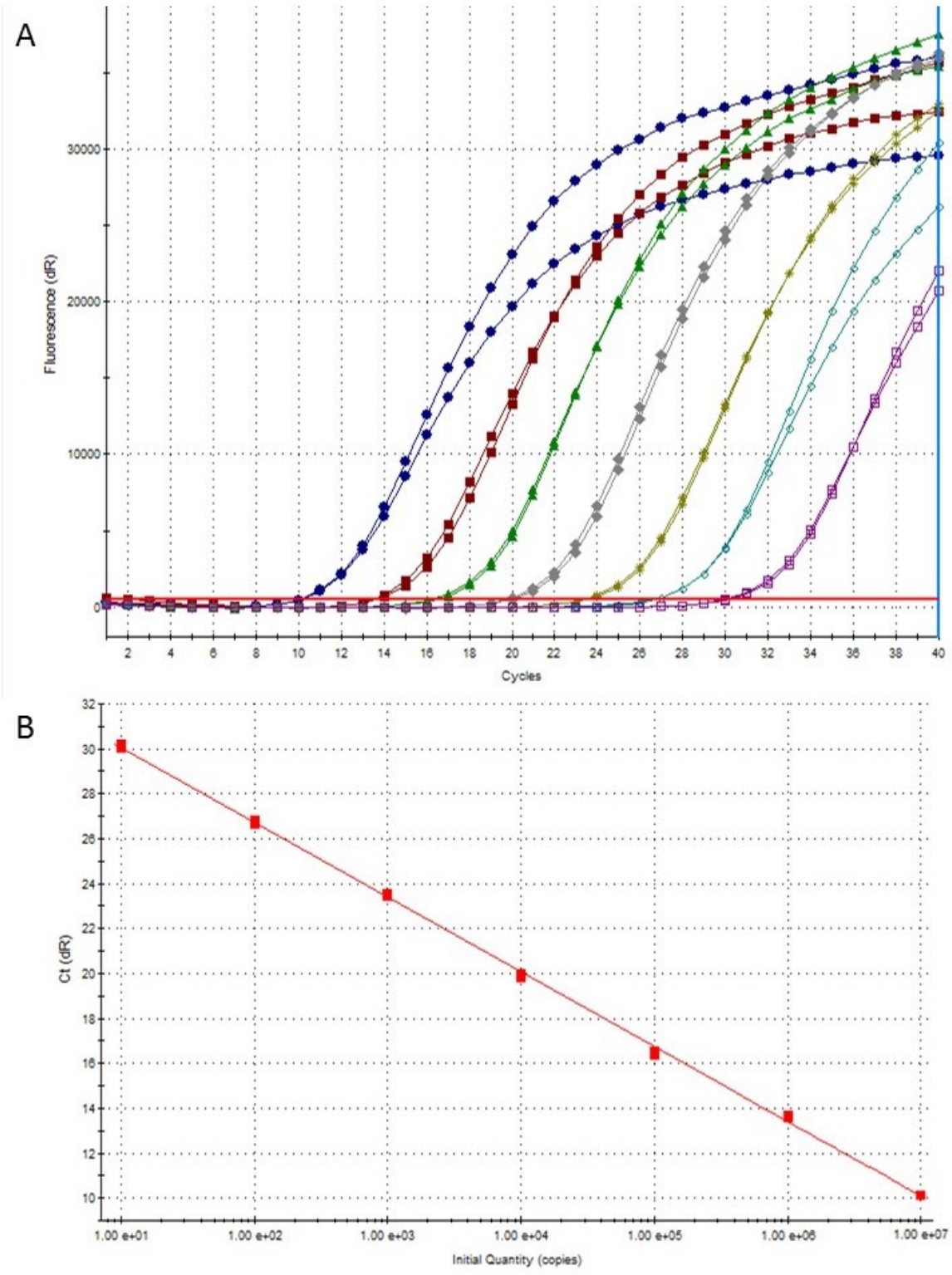


Figure 19 *Amplification plot (Panel A) and standard curve (Panel B) for the Ecoc-8 singleplex qPCR assay using a TEX-labelled TaqMan probe.*
Panel A: *Cycle numbers are plotted on the x-axis and relative fluorescence units are plotted on the y-axis with threshold fluorescence indicated by the red horizontal line and 40 cycle cut-off by the blue vertical line.* ***Panel B:*** *Standard curve as generated from duplicate dilutions over 10E+06-fold. Dilution from the starting quantity is plotted on the x-axis and threshold cycles (Ct) are plotted on the y-axis.*

3.3.1.9 Cross-reactivity of the final M. haemomuris and E. coccoides duplex qPCR assays

No cross-reactivity was detected when challenging the final *M. haemomuris* and *E. coccoides*-specific duplex qPCR assays (Mhm-6 and Ecoc-8) with over 1.0E+05 16S rRNA gene copies (3.2.1.3.3.2) of the respective non-target haemoplasma species (*M. haemomuris* for the *E. coccoides* assay Ecoc-8, and *E. coccoides* for the *M. haemomuris* assay Mhm-6). Positive controls, using 16S rRNA gene amplicon templates, for their respective species-specific duplex qPCR assay resulted in Ct values of 19.06 for *M. haemomuris* and 19.91 for *E. coccoides.*

3.3.1.10 Limit of detection of the final M. haemomuris and E. coccoides duplex qPCR assays

Using a series of ten-fold *M. haemomuris* and *E. coccoides* target DNA dilutions (16S rRNA gene amplicons) in elution buffer (3.2.1.3.3.3), the LoD of the final *M. haemomuris* and *E. coccoides*-specific duplex qPCR assays was determined to be equivalent to one to ten copies per qPCR based on one positive Ct result being obtained out of three replicates (**Table 25**). All three replicates were positive at the one hundred times more concentrated sample, representing the equivalent of 79 *M. haemomuris* 16S rRNA gene copies for the *M. haemomuris*-specific assay and 72 *E. coccoides* 16S rRNA gene copies for the *E. coccoides*-specific assay.

Assay	Molecular weight of amplicon in Daltons (grams)	Measured amplicon concentration prior to dilution (g/ml)	Calculated amplicon copies per reaction prior to dilution	Last detectable dilution of amplicon (>1 in 3 replicates)	Equivalent calculated limit of detection (amplicon copies per reaction)
Mhm-6 duplex	136576.69 (2.27E-19)	5.72E-06	1.26E+11	1.6E+11	0.79
Ecoc-8 duplex	144609.88 (2.40E-19)	5.50E-06	1.15E+11	1.6E+10	7.16

Table 25 *Determination of limit of detection (LoD) for the novel M. haemomuris and E. coccoides-specific qPCR assays. Amplicons of the target-species 16S rRNA gene were diluted 1:160,000 followed by a ten-fold dilution series and LoD defined as the last dilution that gave a positive qPCR result in at least one of three replicates.*

These haemoplasma species-specific duplex qPCR assays were subsequently used to monitor infection kinetics in laboratory mice infected with *E. coccoides* or *M. haemomuris.*

3.3.2 Infection kinetics and protective immunity of murine haemoplasmas

3.3.2.1 Clinical signs and post-mortem examinations

No clinical signs of ill health were noted in the mice other than two showing mild lethargy for about 30 minutes following blood sampling on 9 and 10 dpi after Phase 2 inoculations in the age-matched control group (3.2.2.1.8). The lethargy was attributed to excessive blood loss during the sampling process, rather than parasitaemia, as blood sampling technique did lead to, comparable to other mice, deep incisions over the tail vein in the two affected individuals.

Splenomegaly, defined as splenic weight over 0.4% bodyweight, was documented in all 20 mice at post-mortem examination i.e. in the re-inoculation group, the cross-protection group and aged-matched control group, all killed on 14 dpi after Phase 2 inoculation of *M. haemomuris*-containing blood (3.2.2.1.8) (**Table 26**).

Group	Number of mice examined	Splenic weight range (% bodyweight)	Splenic weight median (% bodyweight)
Re-inoculation group	4	0.48 - 0.56	0.51
Cross-protection group	8	0.66 - 0.89	0.73
Age-matched control group	8	0.67 - 0.92	0.77

Table 26 *Splenic weights (as % bodyweight) in the mice killed 14 days post inoculation with M. haemomuris. Re-inoculation group: Previously infected and recovered from M. haemomuris-infection. Cross-protection group: Previously infected and recovered from E. coccoides infection. Age-matched control group: No previous infection but underwent the same blood sampling regimen as the other experimental groups to account for ongoing blood loss.*

Splenic weight was significantly increased in the cross-protection group (p_{MWU}=0.06) and age-matched control group (p_{MWU}=0.07) (both of these groups had been naïve to *M. haemomuris* infection in Phase 2) compared to the re-inoculation group.

3.3.2.2 Infection kinetics of primary M. haemomuris infection (Phase 1: re-inoculation group)

None of the eight mice in the re-inoculation group had haemoplasma DNA detected by the generic haemoplasma assay on week before entering the experiment.

Only four (all within the same cage) of the eight mice in the re-inoculation group became successfully infected with *M. haemomuris* in Phase 1 of the experiment, following inoculation in Phase 1 with cryopreserved *M. haemomuris*-containing blood (3.2.2.1.5.1), as shown by positive generic haemoplasma qPCR assay (3.2.2.3.1) results. The normalised relative *M. haemomuris* genome copies per µl blood in these four mice in the re-inoculation group are shown in **Figure 20**. An initial lag-phase was observed, followed by a growth phase with a doubling time (dt) of 8.2 hours (calculated from the median *M. haemomuris* genome copies per µl blood per day between 3 and 10 dpi), a median peak of 1.95E+07 *M. haemomuris* copies per µl blood at 11 dpi, followed by a decline and plateau phase (**Figure 20**, **lower panel**). After around two months (60 dpi onwards, see **Figure 20, lower panel**) the *M. haemomuris* genome copies per µl blood started to fluctuate, and this was interpreted as approaching the limits of the PCR detection, but *M. haemomuris* never became completely undetectable on consecutive weeks, and *M. haemomuris* re-inoculation occurred at 153 dpi.

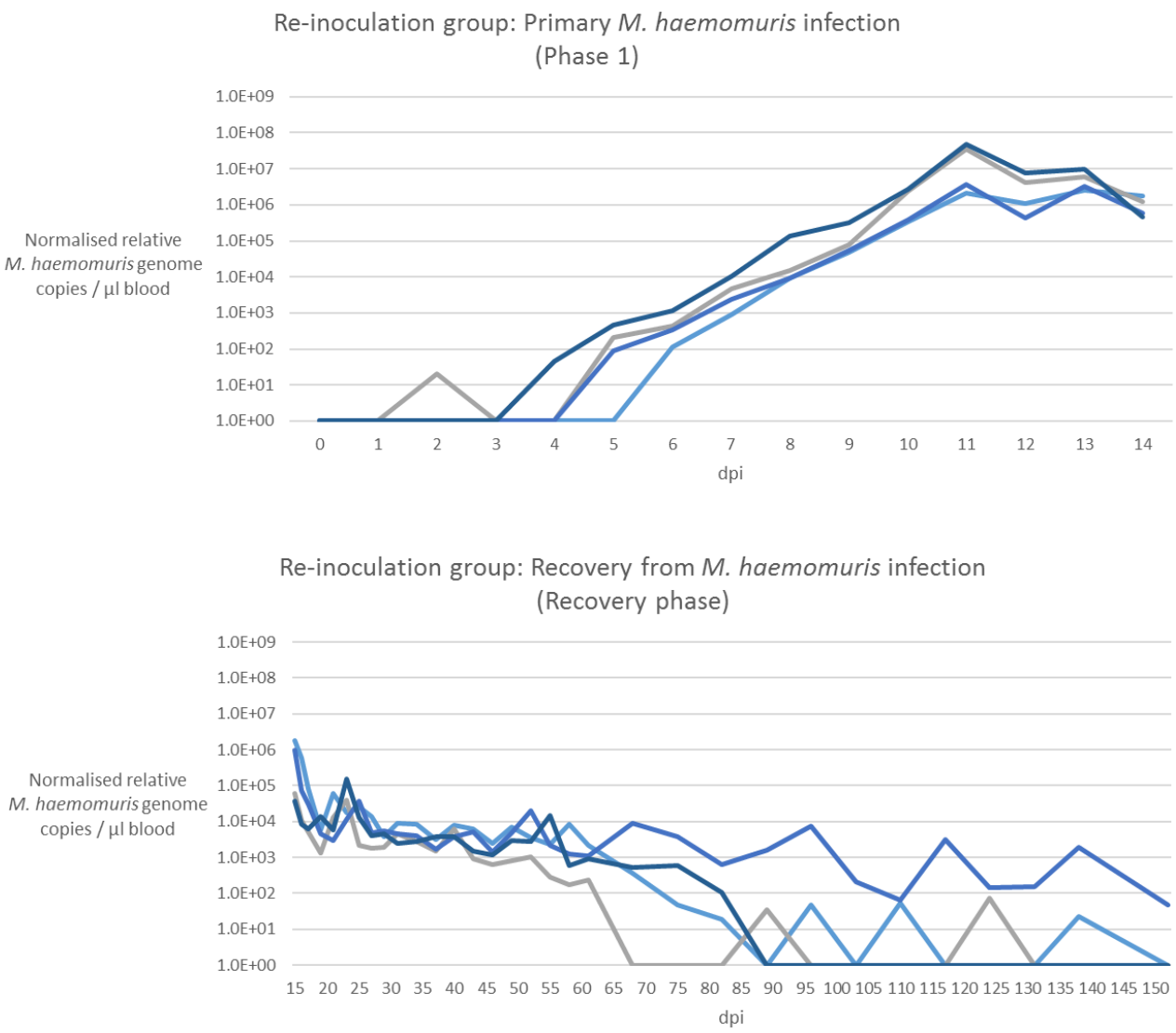


Figure 20 *M. haemomuris genome copies in four of the eight mice in the re-inoculation group (Phase 1: upper panel, Recovery phase: lower panel) showing the infection kinetics of the four naïve mice successfully infected following inoculation with cryopreserved blood containing 2.52E+06 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl of blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily in Phase 1 (upper panel) and at longer intervals in the Recovery phase (lower panel). As zero values cannot be plotted on logarithmic scales, a value of one (1.00E+00) haemoplasma copy per µl blood (and qPCR reaction) is taken as being the equivalent of zero in this and subsequent graphs within this chapter.*

The remaining four mice in the re-inoculation group were monitored for 41 dpi but gave negative generic haemoplasma qPCR assay results at each sampling point, documenting failure of infection with *M. haemomuris*, and were omitted from the remainder of the experiment. However, they were re-inoculated with fresh mouse blood, containing a similar dose of *M. haemomuris* copies as part of a different experiment and showed similar infection kinetics curves as observed in the successfully infected mice of the re-inoculation group (data not shown), suggesting inoculum failure rather than immunological protection during the failed Phase 1 inoculation.

3.3.2.3 Infection kinetics of primary M. haemomuris infection (Phase 2: age-matched control group)

None of the eight mice in the age-matched control group had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment and from blood samples taken immediately prior to Phase 2 inoculations (to ensure the separately housed mice had maintained haemoplasma-negative status).

The eight mice in the age-matched control group became successfully infected with *M. haemomuris* in Phase 2 of the experiment, following inoculation in Phase 2 with freshly collected *M. haemomuris*-containing blood (3.2.2.1.5.2), as shown by positive *M. haemomuris*-specific qPCR assay (3.2.2.3.2) results. The normalised relative *M. haemomuris* genome copies per µl blood in the age-matched control group are shown in **Figure 21**. Compared to the primary *M. haemomuris* infection in the re-inoculation group (3.3.2.2), the infection kinetics in the age-matched control group showed similar median peak values of 2.85E+07 *M. haemomuris* genome copies per µl blood at 10 dpi but demonstrated an early and prolonged growth phase with a dt of 16.6 hours calculated from the median *M. haemomuris* genome copies per µl blood per day between 3 and 10 dpi, but a dt of only 7.7 hours between 0 and 7 dpi, and no detectable lag-phase.

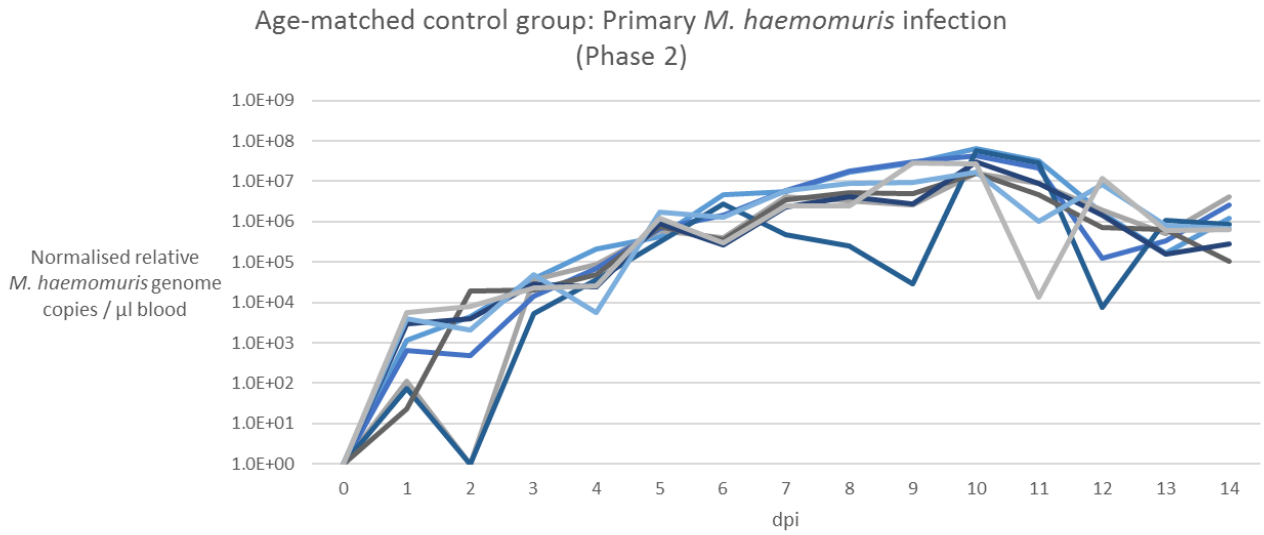


Figure 21 *M. haemomuris genome copies in the eight mice in the aged-matched control group (Phase 2), showing the infection kinetics of eight naïve mice successfully infected following inoculation with freshly obtained blood containing 1.90E+04 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily in Phase 2.*

The different infection kinetics of the primary *M. haemomuris* infection in the age-matched group in Phase 2 to the re-inoculation group in Phase 1 were assessed by modified comparative statistical analysis. To eliminate the effect of an absent lag-phase and synchronise infection kinetics curves based on the onset of bacteraemia, Mann-Whitney U statistics were performed on AUC values from 0 to 14 dpi for the age-matched group in Phase 2 (that received fresh inocula, see **Figure 21**) and from 3 to 17 dpi for the re-inoculation group in Phase 1 (that received cryopreserved inocula, see **Figure 20, upper panel**). There was no significant difference in *M. haemomuris* genome copy AUC values over these two 14-day periods (p_{MWU}=0.089).

3.3.2.4 Infection kinetics of primary E. coccoides infection (Phase 1: cross-protection group)

None of the eight mice had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All eight mice successfully became infected with *E. coccoides* in Phase 1 of the experiment, following inoculation in Phase 1 with cryopreserved *E. coccoides*-containing blood, as shown by positive generic haemoplasma qPCR assay (3.2.2.3.1) results. The normalised relative *E. coccoides* genome copies per µl blood in the eight mice in the cross-protection group are shown in **Figure 22**. There was a brief growth phase, a peak and then subsequent decline in *E. coccoides* genome copies per µl blood, and negative generic haemoplasma qPCR results started to occur as early as 10 dpi; thereafter the *E. coccoides* genome copies per µl blood continued to be low or were undetectable until cross-challenge with *M. haemomuris* occurred at 153 dpi (see later, not shown in **Figure 22**).

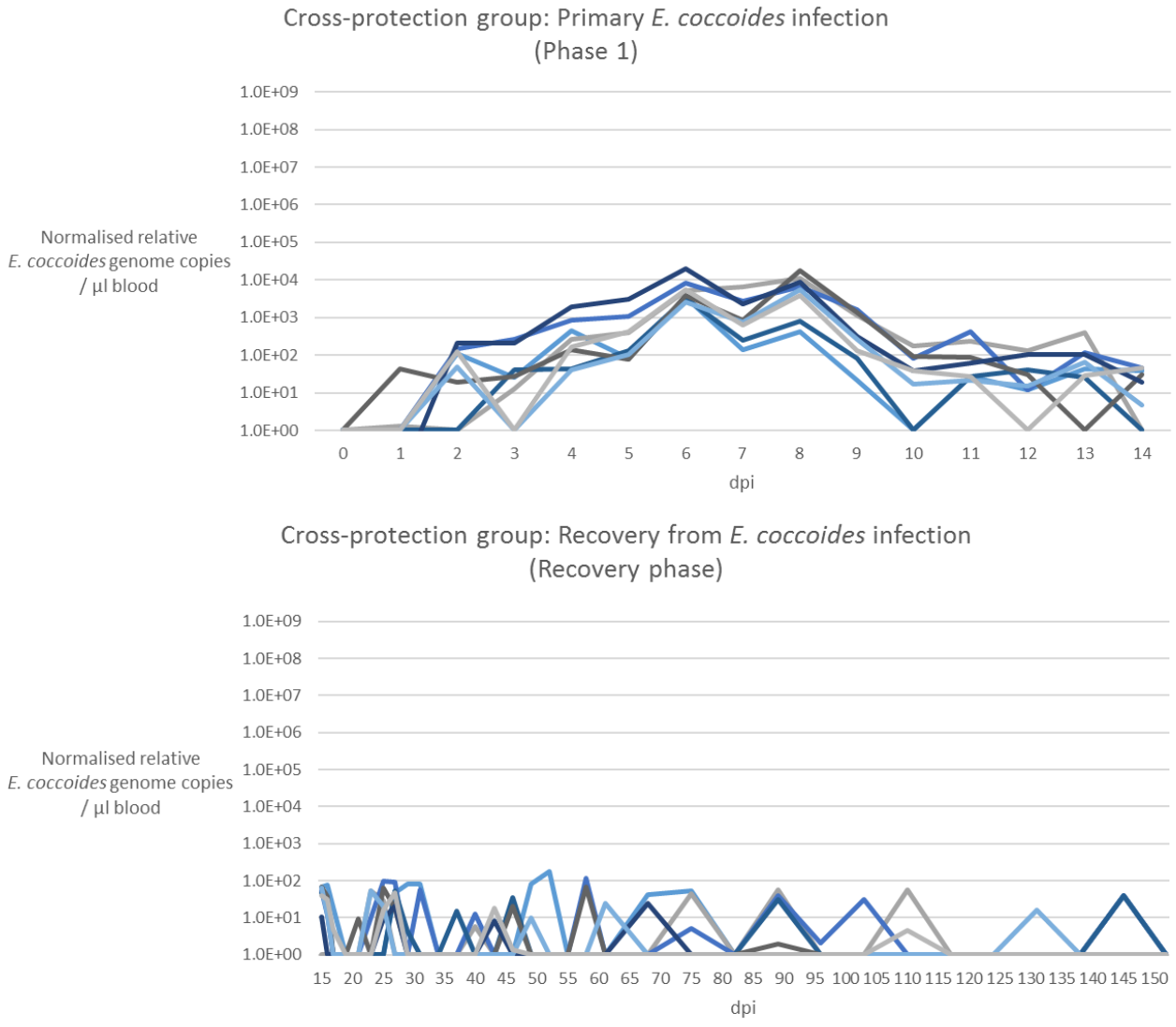


Figure 22 *E. coccoides genome copies in the eight mice in the cross-protection group (Phase 1: upper panel, Recovery phase: lower panel) showing the infection kinetics of the eight naïve mice successfully infected following inoculation with cryopreserved blood containing 4.25E+06 E. coccoides copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and normalised relative E. coccoides genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily in Phase 1 (upper panel) and at longer intervals in the Recovery phase (lower panel)*

Mann-Whitney U statistics were performed on AUC values from 0 to 14 dpi to show that the *E. coccoides* genome copies per µl blood in this primary *E. coccoides* infection in the cross-protection group in Phase 1 were significantly lower than both the *M. haemomuris* genome copies per µl blood seen during primary infection in the re-inoculation group in Phase 1 (p_{MWU}=0.007) (3.2.2.1.6.1, kinetics given in **Figure 20**) and also the *M. haemomuris* genome copies per µl blood seen during primary infection in the age-matched control group in Phase 2 (p_{MWU}=0.001) (3.2.2.1.8.1, kinetics given in **Figure 21**).

No re-inoculation with *E. coccoides* was performed as it was not part of the experiment (3.2.2.1.4.1).

3.3.2.5 Partial protection from re-infection with M. haemomuris following previous M. haemomuris infection (Phase 2: re-inoculation group)

Normalised relative *M. haemomuris* genome copies per µl blood in the four mice in the re-inoculation group, that had been previously infected and partially recovered from *M. haemomuris* infection inoculated in Phase 1, following repeat inoculation with *M. haemomuris* in Phase 2 on day 153 dpi using freshly collected blood (from amplifier mice, 3.2.2.1.5.2) without cryopreservation, are shown in **Figure 23**; 153 dpi is represented as 0 dpi in the graph for simplicity. The mice were monitored for infection using the *M. haemomuris*-specific qPCR assay (3.2.2.3.2). The *M. haemomuris* genome copies per µl blood during the Recovery phase, between Phase 1 and Phase 2, are shown in **Figure 20** above.

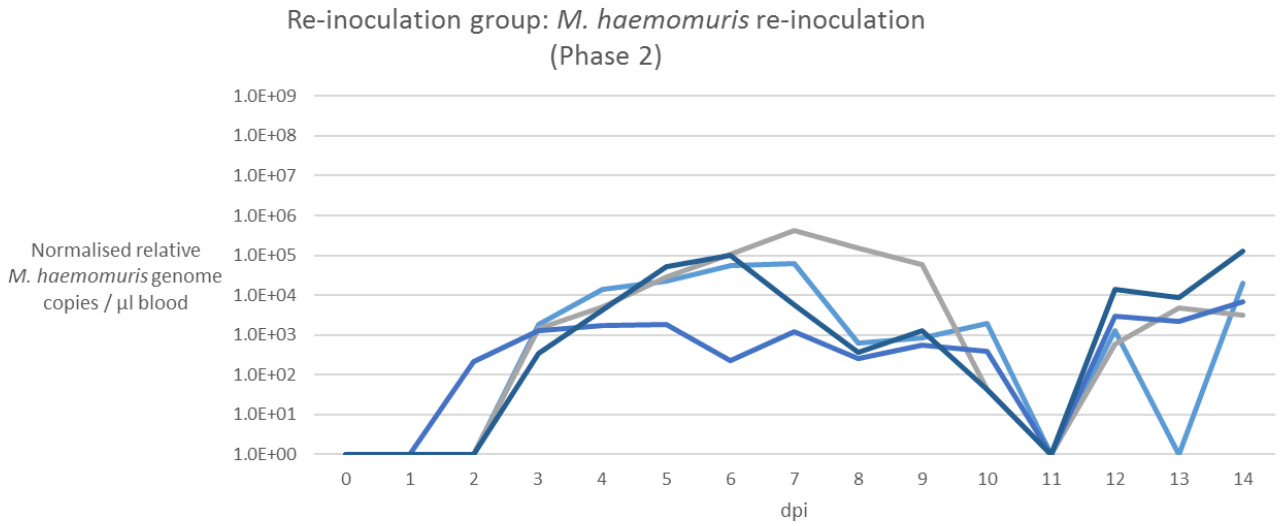


Figure 23 *M. haemomuris genome copies in the four mice in the re-inoculation group (Phase 2), showing the infection kinetics of four mice previously recovered from M. haemomuris following re-inoculation with freshly obtained blood containing 1.90E+04 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily in Phase 2.*

The four mice previously infected with *M. haemomuris* in the re-inoculation group demonstrated significantly lower (p_{MWU}=0.007) *M. haemomuris* genome copy AUC values in the 14-day period of Phase 2 (**Figure 23**) than those in the eight mice in the age-matched control group in the corresponding period of Phase 2 (**Figure 21**); both these groups were infected with *M. haemomuris* using identical inocula. This suggests partial protection from re-infection with *M. haemomuris* in the re-inoculation group.

3.3.2.6 Lack of protection from M. haemomuris infection following previous E. coccoides infection (Phase 2: cross-protection group)

Normalised relative *M. haemomuris* genome copies per µl blood in the eight mice in the cross-protection group, that had been previously infected and partially recovered from *E. coccoides* infection inoculation in Phase 1, following inoculation with *M. haemomuris* in Phase 2 on day 153 dpi using freshly collected blood (from amplifier mice, 3.2.2.1.5.2) without cryopreservation, are shown in **Figure 24**; 153 dpi is represented as 0 dpi in the graph for simplicity. The mice were monitored for infection in Phase 2 using the *M. haemomuris*-specific qPCR assay (3.2.2.3.2) and the *E. coccoides*-specific qPCR assay (3.2.2.3.2). The *E. coccoides* genome copies per µl blood during the Recovery phase, between Phase 1 and Phase 2, are shown in **Figure 22**, **lower panel** above.

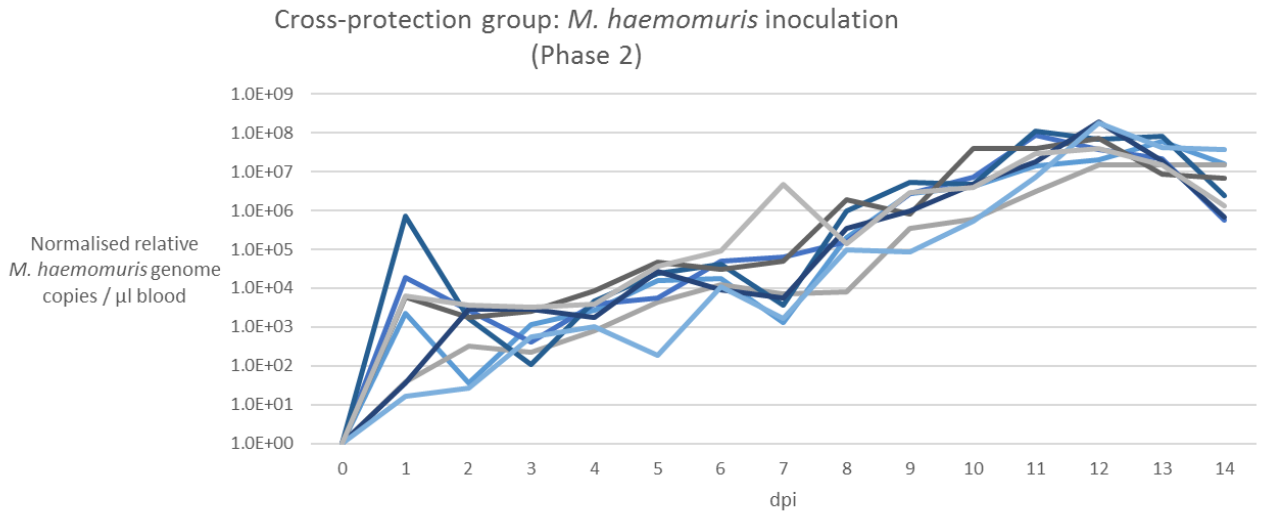


Figure 24 *M. haemomuris genome copies in the eight mice in the cross-protection group (Phase 2), showing the infection kinetics of eight mice previously recovered from E. coccoides infection following inoculation with freshly obtained blood containing 1.20E+04 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily in Phase 2.*

The eight mice previously infected with *E. coccoides* in the cross-protection group showed *M. haemomuris* infection kinetics in Phase 2 (**Figure 24**) that included an initial median peak of 6.99E+05 *M. haemomuris* genome copies per µl blood at 1 dpi, followed by a growth phase and further median peak of 5.35E+07 *M. haemomuris* genome copies per µl blood at 12 dpi. This peak was two days later than the 2.85E+07 10 dpi peak seen in the age-matched control group (**Figure 21**).

The eight mice previously infected with *E. coccoides* in the cross-protection group demonstrated significantly higher (p_{MWU}=0.024) *M. haemomuris* genome copy AUC values in the 14-day period of Phase 2 (**Figure 23**) than those in the eight mice in the age-matched control group in the corresponding period in Phase 2 (**Figure 21**). The dt of *M. haemomuris* in the cross-protection group was 13.3 hours between 0 and 7 dpi, and 17.7 hours between 3 and 10 dpi. There was no apparent re-emergence of *E. coccoides* infection (i.e. negative results on the *E. coccoides*-specific qPCR assay) throughout Phase 2.

3.4 DISCUSSION

3.4.1 Successful development of qPCR assays specific to *M. haemomuris* and *E. coccoides* and duplexed with a murine GAPDH internal control

We have, for the first time, developed and validated and duplexed (with a GAPDH internal control assay) qPCR assays for the detection of infection with *M. haemomuris* and *E. coccoides* in mice. These species-specific assays are essential when investigating possible cross-protection of *E. coccoides* to *M. haemomuris* infection, and when infection with both these haemoplasma species is possible in a host species. The inclusion of the GAPDH internal control was important to help rule out errors or variation in DNA extraction or PCR amplification to avoid false-negative results.

As *M. haemomuris* and *E. coccoides* cannot be cultured *in vitro*, no samples of known colony forming units (CFU) could be generated to use as standards in qPCR to enable deduction of the exact number of haemoplasma copies present in samples. The use of 16S rRNA gene amplicons as an alternative target to allow calculation of haemoplasma copies in the samples assumes that only one 16S rRNA gene exists per haemoplasma genome; this has been demonstrated for all fully sequenced haemoplasmas (74-80, 82, 83) except for *Mycoplasma ovis* (81), in which two copies per genome were found. Another assumption that is made in these calculations when effectively equating one genome copy with one CFU is that there is 100% viability of the detected haemoplasma copies, which cannot be proven in the absence of *in vitro* cultivation. However, the use of plasmids containing the target region of a PCR assay is a widely accepted method to allow qPCR assay validation (308), and so we considered the use of PCR amplicons a suitable substitution for plasmids. However, the use of PCR amplicons is potentially less accurate than using actual CFUs or plasmids encoding the target region to determine a qPCR assay's sensitivity due to the smaller size of PCR amplicons and thus errors that can result in measuring DNA content could contribute to large discrepancies in calculated amplicon copy numbers.

The initial duplex conditions (3.2.1.3.2.2) in the qPCRs to amplify both the internal control GAPDH and specific rodent haemoplasma species resulted in higher Ct values for the haemoplasma amplification compared to the singleplex qPCR assay for the haemoplasma species alone, despite the use of the same conditions and dilution series. This was assumed to be due to competitive inhibition of the haemoplasma qPCR within the duplex qPCR by the internal control qPCR. The primer and probe concentrations of the internal control qPCR were subsequently reduced until inhibition was no longer seen. The reduction in primer and probe concentrations for the internal control qPCR resulted in flattening of the amplification curves and a small increase in Ct when compared to the original results (data not shown). This was deemed unimportant as quantification of mouse gDNA was not the purpose of the assay and normalisation of haemoplasma genome copies to mouse GAPDH gene copies is not affected by curve shape or higher Ct values, as long as detection is reliable and delivers repeatable results. Similar problems have been seen with other duplex qPCR assays that target an abundant internal control gene (e.g. GAPDH), Barker, Tasker (187) and Peters, Helps (100).

Both duplex qPCR assays were able to detect between one and ten copies of either *M. haemomuris* or *E. coccoides*, which is of comparable sensitivity to the currently used qPCR assays for feline and canine haemoplasmas (100, 149, 187). In clinical laboratories, the last detectable dilution of template DNA is often reported as a qPCR assay's sensitivity or LoD, although this definition can be misleading as the practical LoD for any qPCR assay is about three to four copies of template DNA per reaction (309). A more general definition, accounting for the number of replicates used to detect low-quantities of target DNA, and reporting a probability rather than a number of target DNA copies,

is given by the Clinical and Laboratory Standards Institute (310), and may better reflect the true LoD in a laboratory setting: the LoD is defined as the lowest amount of analyte in a sample that can be detected with a given probability. It is important to note that this definition does not include any information on the assay's ability to quantify this detection. The limit of quantification (LoQ) is defined as the lowest amount of measurand in a sample that can be quantitatively determined with given acceptable precision and acceptable accuracy, under given experimental conditions (311). Whilst determination of LoQ was not attempted for the current study, our reported LoD of one to ten haemoplasma genome copies does need to be interpreted in conjunction with the fact that the assays were only able to detect one to ten haemoplasma genome copies in one out of three replicates. Guidelines for the determination of LoD in non-culturable targets call for at least six replicates to be performed over a ten-fold dilution series (308), so that the LoD could then be stated as the lowest genome copy equivalent (highest dilution) that yielded positive results over all six replicates. However, the same resource also reports any LoD lower than 20 genome equivalents per reaction to be ideal, which is close to what could be extrapolated from our dataset (**Table 25**). However, more replicates and assessment of target molecule copies per reaction that are less susceptible to error accumulation due to small amplicon size (i. e. using plasmids) would be required to report LoD in agreement with more stringent laboratory standards than currently applied to our research assays. Another limitation of our evaluation of assay sensitivity (defined as LoD) is that the dilution series was performed in elution buffer rather than in a background of mouse DNA, and background DNA could have interfered with assay performance at low target copies per reaction by unspecific product formation or competition of the mouse GAPDH assay for reaction substrates. However, no interference of the assays was demonstrated with the use of the optimised duplex conditions (using a background of mouse DNA) and no non-specific reactions with mouse DNA were demonstrated during the evaluation with SYBR Green 1, making it very unlikely that we would have encountered different LoD results when using a background of mouse DNA.

No cross-reactivity was found between the *E. coccoides* and *M. haemomuris* species-specific qPCR assays when they were challenged with over 1.0E+05 target amplicon copies of the respective non-target species. This is in full agreement with accepted guidelines that state that at least 1.0E+03 genome copies of non-target species should not trigger cross-reactivity (308). However, cross-reactivity to other non-target species was only assessed *in silico* and would need to be evaluated to further confirm specificity of these species-specific qPCR assays, especially if, for example, other haemoplasma species were found to infect mice.

Validation guidelines (308) also exist for multiplex (i.e. duplex) qPCR assays and include having a linearity of dilution with a correlation coefficient of greater than 0.98 and a reaction efficiency of 80 to 120 % or with a slope of -2.9 to 3.9; all of these criteria were met by our *E. coccoides* and *M. haemomuris* species-specific qPCR assays as they exhibited correlation coefficients and reaction efficiencies (*E. coccoides* assay: 0.999, E=99.6 %; *M. haemomuris* assay: 0.999, E=98.0 %) close to describing theoretical exponential amplification of target copies during the qPCR reaction. However, both the *M. haemomuris* and *E. coccoides* species-specific qPCR assays were only evaluated for use in blood. These assays would need to be re-evaluated before they can be used in different situations e.g. if being used to detect haemoplasma DNA in vector samples (e.g. tick, flea) or different tissues (e.g. spleen, liver) compared to blood.

3.4.2 Monitoring of infection kinetics of *M. haemomuris* and *E. coccoides* in C57BL/6 mice under experimental conditions and protection from re-infection with *M. haemomuris*

We have, for the first time, quantitatively monitored *M. haemomuris* and *E. coccoides* infection in mice, which is a crucially important step in the establishment of a laboratory mouse model of

haemoplasma infection. Cohen, Shemesh (34) have recently described the infection dynamics of a '*M. haemomuris*-like haemoplasma' in wild-caught, experimentally infected gerbils with a blood sampling interval of approximately two weeks. We observed a similar infection kinetics pattern to them, with a lag-, peak-, decline- and plateau-phase for *M. haemomuris* but not *E. coccoides* infection. The peak *M. haemomuris* genome copies per µl of blood seen in the current studies describing primary *M. haemomuris* infection (3.3.2.2 and 3.3.2.3) was approximately 100-fold higher than that observed by Cohen, Shemesh et al. in gerbils, but this could be due to the infrequent blood sampling in the gerbil study missing short-lived peaks in bacteraemia. In cats, a peak bacteraemia of approximately 1.0E+06 genome copies per µl blood occurs after *M. haemofelis* and *'Ca.* M. haemominutum' infection, but levels do not significantly decline before entering a plateau-phase following naïve infection (89, 107, 136). Infection of cats with '*Ca.* M. turicensis' results in a peak bacteraemia of 1.0E+03 per µl blood, with the organism becoming undetectable after approximately three months (124), which resembles the *E. coccoides* infection kinetics seen in the current study in mice, and may reflect the phylogenetical relationship between these two haemoplasmas, within the HF-group of haemoplasmas (see phylogeny presented in Chapter 2, **Figure 5**). Infection kinetics have only been described in one more of the veterinary relevant haemoplasmas, *M. suis* (120). Peak *M. suis* bacteraemia levels of over 1.0E+08 per µl blood have been described in experimental infection using splenectomised piglets, also showing a less pronounced decline prior to establishment of a plateau-phase as it is seen in feline haemoplasmas (89, 136).

One major important finding in the current study is that partial protection from re-infection with *M. haemomuris* was observed in C57BL/6 mice; demonstrated by significantly lower levels of bacteraemia in mice that had been previous infected with *M. haemomuris* compared to those naïve to infection by undergoing *M. haemomuris* infection for the first time. Protective immunity to haemoplasma infection has previously been demonstrated for *M. haemofelis* (107) and '*Ca.* M. turicensis' in cats (124), both species of which are able to induce anaemia in immunocompetent cats, although *M. haemofelis* (26) appears to be much more pathogenic than '*Ca.* M. turicensis' (38). In the current study, we did not monitor haematological variables and so we are not able to document whether the observed protection from high levels of bacteraemia in *M. haemomuris* infection also protected the mice from developing anaemia or erythrocytic osmotic fragility, as is observed in *M. haemofelis* (123) and '*Ca.* M. turicensis' (124) infection in cats. Although no obvious clinical signs of ill health were noted in the mice following *M. haemomuris* infection, which might have been expected if significant anaemia had resulted, we cannot rule out that anaemia or other changes in erythrocytic variables occurred as mild clinical signs, such as pallor or mild lethargy, are extremely easy to overlook in small rodents (312). One might speculate that the lethargy seen after the blood sampling procedure in two mice, as it occurred around peak bacteraemia in primary infection, could have represented such an occurrence, although increased bleeding from the sampling site was seen and attributed to the sampling procedure at the time.

In the current study we used an inbred strain of mouse from the same supplier, of the same age, housed in the same facility and subjected to all the same experimental methods except for the haemoplasma species used for inoculation. Thus, we were able to minimise any effects of the source of the mice, their age, housing etc. on variation of the immune response. This has not been achieved in the same way in some of the published feline studies, e.g. in the study demonstrating protective immunity to *M. haemofelis* infection, the naïve cats inoculated with *M. haemofelis* were juvenile (seven months old) whereas the previously *M. haemofelis*-infected cats were much older, having been kept in the research establishment for seven years, and under different housing conditions (107). Variation such as this in the feline study demonstrates the need for a mouse model where

experimental conditions can be more easily closely controlled and allow investigations in a more cost-effective manner due to the lower price of purchase and maintenance costs of mice.

We were able to show that no cross-protection from *M. haemomuris* infection occurs in mice previously infected with *E. coccoides* as *M. haemomuris* was still able to infect and establish 'naïve-like' infection kinetics in mice that had previously recovered from *E. coccoides* infection. Lack of cross-protection between haemoplasma species has been experimentally demonstrated for *M. haemofelis* following previous recovery from '*Ca.* M. turicensis' infection (295), and the concept of lack of cross-protection is supported in many host species due to the occurrence of multiple haemoplasma species infections in individual canine and feline hosts as commonly reported (for example, by Fujihara, Watanabe (294), Peters, Helps (100) or Filler (98)). However, co-infection with *M. haemomuris* and *E. coccoides* has not been demonstrated in rodents. This may be due to the lack of species-specific PCR assays available to enable detection of more than one haemoplasma species in a host (35, 36, 53). In fact, the current study demonstrated a higher number of *M. haemomuris* genome copies per µl blood in the cross-protection group, following *E. coccoides* infection, than in naïve infection with *M. haemomuris* in the re-inoculation and age-matched control groups. The latter corresponds to findings in cats (295), where signs of enhancement of *M. haemofelis* infection were seen in cats previously infected with '*Ca.* M. turicensis'. And although different inocula (different donor mice and respective *M. haemomuris* inoculum dose) were used for inoculation of *M. haemomuris* in the cross-infection and the age-matched control groups, the *M. haemomuris* genome copies inoculated do not readily explain the greater increase in genome copies per ul of the cross-protection mice (who received 2.40E+02 *M. haemomuris* copies) compared to the age-matched control mice (who received 3.80E+02 *M. haemomuris* copies).

An even more pronounced difference between *M. haemomuris* genome copies was seen in naïve infection in the younger mice in Phase 1 (re-inoculation group) and naïve infection in the 'aged' mice in Phase 2 (age-matched control group), where the young mice exhibited a lag-phase that was not observed in primary infection of the 'aged' mice. The more rapid onset of infection in the 'aged' group (Phase 2, age-matched control group) was attributed to the difference in *M. haemomuris* inocula used (cryopreservation and dilution) although it is possible that age of inoculated mice might have been a contributing factor. The inoculum used for Phase 1 (re-inoculation group) was cryopreserved and it appeared to be less infective than expected as only four of the eight mice in the re-inoculation group became successfully infected with *M. haemomuris* initially. It was speculated that cryopreservation with 20% DMSO (v/v) and/or dilution in PBS may have reduced *M. haemomuris* viability significantly as the inoculum contained very high *M. haemomuris* genome copy numbers (2.52E+06). A similar complication was suspected in studies by Hicks, Willi (107) when using a low-dose *M. haemofelis* infection model following cryopreservation of inocula with 20% DMSO (v/v). In Phase 2 of the experiment, only fresh inocula were used and the age-matched control group received the same inocula and *M. haemomuris* doses as the re-inoculation group, hence ruling out reduced inoculum viability as a cause for the observed partial protection from re-infection with *M. haemomuris*.

Following primary infection with *E. coccoides*, mice were not re-challenged with the same organism to see if prevention of re-infection occurred with *E. coccoides*, nor did we attempt to inoculate *E. coccoides* into mice previously infected with *M. haemomuris* to see if cross-protection to *E. coccoides* occurred. These studies would have been interesting to perform but were not carried out due to cost and ethical concerns. Ultimately, our aim was to select and describe a laboratory animal model of haemoplasma infection in a veterinary relevant species, and our results suggested that the *M. haemomuris* model was most appropriate for this, as *M. haemomuris* showed prolonged and

high-level bacteraemia, and protection from re-infection, akin to the changes seen with the feline pathogenic haemoplasma *M. haemofelis*, suggesting its suitability for further investigations.

We observed fluctuations of *M. haemomuris* and *E. coccoides* genome copies per µl blood at peak bacteraemia and at late stages of infection (around 80 dpi for *M. haemomuris* and as early as 10 dpi for *E. coccoides*). Dramatic fluctuations in haemoplasma genome copies per µl blood over periods of just a few days are a feature of *M. haemofelis* infection in cats around peak bacteraemia, but may also occur when the number of genome copies per µl blood are close to the LoD (89, 107). Cycling of haemoplasma copy numbers, e.g. a 1.0E+06-fold difference in haemoplasma copy number over just 2 days, has been demonstrated during *M. haemofelis* infection (89) but was not seen during peak bacteraemia in mice in the current study. The observed low-level fluctuation of *M. haemomuris* and *E. coccoides* genome copies per µl blood were more likely due to stochastic effects during sample processing and might have disappeared if more replicate qPCRs per timepoint had been done, or higher blood volumes used for DNA extraction at each time point. This is an important consideration for the planning of studies evaluating clearance of infection or other applications where negative PCR results must be confirmed. Clearance from *M. haemofelis* in cats after consecutive antibiotic treatment was recently demonstrated using triplicate or quadruplicate qPCRs per negative sample (135).

We noted splenomegaly in all mice in all groups at post-mortem examination, although splenic weight was significantly greater in mice that had undergone primary *M. haemomuris* infection than in mice that had undergone *M. haemomuris* re-infection. One possible explanation for this is that the spleens were not activated (for haemolysis, for example, or in immunological reactions to remove *M. haemomuris*) as much when lower *M. haemomuris* copy numbers were present in the blood. As no haematological variables or *M. haemomuris* copy numbers per mg of spleen were monitored in the current study, it is hard to speculate further on splenic activation. We did, however, account for the fact that splenomegaly could have been introduced by the effect of repeated blood loss during blood sampling, as the age-matched control group all underwent the same blood sampling protocol in Phase 1 and in the Recovery phase as the other groups. However, we did not include a healthy control group from within the same study population for the mice exsanguinated at the end of the experiment, 14 days after Phase 2 inoculations. Hence it remains unclear whether the lesser splenomegaly seen in the re-inoculated group was indeed clinically significant. We also did not assess the occurrence of splenomegaly during primary *E. coccoides* infection or at any other timepoint than 14 dpi with *M. haemomuris*, so further studies would be needed to further investigate splenomegaly over the course of rodent haemoplasma infections.

3.4.3 Limitations of the current study and suggested future work using the mouse model of haemoplasma infection

Limitations of the newly validated duplex qPCR assays targeting the *M. haemomuris* and *E. coccoides* 16S rRNA genes have been mentioned above. Both assays were shown to have the required sensitivity and specificity for use in a mouse model of *M. haemomuris* and *E. coccoides* infection, but further work on inter- and intra-assay variability (robustness and LoQ), and their use in different types of samples, are ideally required.

In the current study we have established a surrogate (see 1.5.2) mouse model of haemoplasma infection, using two different rodent haemoplasma species, *M. haemomuris* and *E. coccoides*, and the 'fit-for-purpose' scoring system of animal models as proposed by Denayer, Stöhr (313) that accounts for the research objective (i.e. monitor infection kinetics rather than assess therapy or measures of prevention) and the fact that our aim was to establish and monitor infection

irrespective of clinical disease. We did however, encounter problems with infecting mice using PBS-diluted and DMSO-cryopreserved inocula. No comparative studies into haemoplasma cryopreservation have been carried out but DMSO is known to be cytotoxic during prolonged exposure at warmer temperatures (259). We thus subsequently adjusted our protocols to use 10% v/v glycerol, instead of DMSO as a cryopreservative, as glycerol is less likely to by cytotoxic at room temperature or above (259). We also changed our experimental protocols to use amplifier mice in order to generate larger numbers of viable haemoplasmas for standardised inoculation of experimental mice.

We have used this surrogate mouse model of haemoplasma infection to demonstrate partial protection from *M. haemomuris* re-infection and the lack of cross-protection through previous *E. coccoides* infection. We have further selected *M. haemomuris* to be the most appropriate rodent haemoplasma pathogen to model *M. haemofelis* infection in cats based on their similar infection kinetics and induction of splenomegaly. However, before the use of this model in studies assessing therapy or vaccination, we will need to document the clinicopathological features of *M. haemomuris* infection in mice and establish their relatedness to haemoplasmosis in other veterinary species of social (e.g. cats) or economic (e.g. pigs) importance. Such a comparison of clinicopathological changes will help assess the model's 'face validity' or the similarity in biology and symptoms between the animal model and the modelled disease (313) and is important in model validation, however, it may be adjusted to only include a subset of signs.

Future studies will therefore aim to include the collection of some clinicopathological data, such as haematology, over the course of *M. haemomuris* infection, as this was not possible in the studies described in this chapter due to the limitation of quantity of blood that could be collected safely (up to a maximum of 10% total blood volume per day or 25% total blood volume per 4 consecutive weeks, accounting for 116µl per day or 290µl over 4 weeks) from the mice. Additionally, as we have demonstrated that partial protective immunity against *M. haemomuris* infection does occur in mice, immunological changes associated with the development of immunity should now be further investigated.

4 FURTHER CHARACTERISATION OF THE MOUSE MODEL OF HAEMOPLASMA INFECTION

4.1 INTRODUCTION

4.1.1 Rationale for characterising the immune response to *M. haemomuris* infection in the mouse model of haemoplasma infection

Following observations of protective immunity against re-infection with the same haemoplasma species in cats (107, 124) and mice (see Chapter 3), the development of a vaccine to protect from infection and/or clinical haemoplasmosis appears possible. The need for a vaccine is highlighted by the difficulty in being able to consistently clear haemoplasma infections from chronic carriers; only very recently has a possible protocol to achieve this been suggested in cats, and this comprised a long treatment protocol using two different antibiotics (135). In pigs, clearance of haemoplasma infection is economically challenging and has not focused on outcomes in individual pigs, but on removal of carrier pigs from the group and employment of sanitary methods that avoid iatrogenic transmission via shared needles etc. during husbandry measures (120). However, generally, further research into haemoplasma vaccine development is still hindered by the lack of an *in vitro* cultivation system for the haemoplasmas. Without the ability to grow haemoplasmas *in vitro*, our ability to investigate haemoplasma-host-interactions, including the molecular characterisation of protective immunity, is still greatly limited.

Cytokines are small proteins that are biologically active as signalling molecules, either between leukocytes (interleukins; IL) or as chemotactic attractants (chemotactic cytokines; chemokines) of a variety of immunologically active cells (288). In a variety of host species and pathogens, certain cytokine profiles are known to be associated with a higher risk of infection (314) or a higher severity of disease or even death (315). Conversely other cytokine combinations have been identified to suggest protection from infection with a variety of extracellular (316) or intracellular pathogens (317).

Knowledge of the nature of the immune response to a specific pathogen is required to guide decision making in preventative (i.e. vaccination) strategies against infection and to protect from clinical disease. Different branches of the immune system that are involved in the formation of protective immunity can potentially be addressed with vaccine design, e.g. by adding adjuvants that mimic or attract certain cytokines (318, 319) and hence induce a pronounced and lasting immune response. The general principles of the mammalian immune response (largely based on the mouse) and our current knowledge of immunological mechanisms involved in haemoplasma infection are outlined in the sections below.

4.1.1.1 Patterns in immunology

4.1.1.1.1 Innate and acquired response to infection

Mammals have developed a variety of mechanisms to fight pathogens and prevent infection. Broadly, these can be split into innate and acquired immunity.

Innate immunity comprises mechanisms encoded in the germline of the host, such as phagocytic cells, natural killer (NK) cells or the presence of substances in the blood to protect against infection, i.e. lysozyme, basic polypeptides and the complement complex (320). The cellular component of innate immunity (omitting mechanical barriers such as skin or gut lining) is mainly comprised of

granulocytic cells (neutrophils, eosinophils, basophils, monocytes, macrophages and specialised phagocytic cells) that proliferate in the presence of pathogen (e.g. bacterial) invasion and tissue injury and can also be monitored in the bloodstream to monitor clinical progression of disease (321). Phagocytic cells also produce cytokines to recruit further cells and guide the subsequent acquired immune response (322). Many of these innate defence mechanisms account for the species-specificity of certain pathogens as they acquire specific ways to evade from or even invade the host's defence (323).

Acquired immunity is not directly encoded in the germline but based on constant variation of surface receptors to bind potential pathogens. This high variability, however, takes time to develop as it needs constant monitoring to eliminate self-reactiveness (324). Hence, an acquired immune response does not develop until exposure to an antigen, either naturally or via vaccination, and is primarily mediated by antigen-specific receptors expressed by lymphocytes (T-cell receptors and immunoglobulins). Antigens are typically proteins or large polysaccharides that are specific to the pathogen but cross-reactivity can occur (325). Acquired immunity can be split into two broad subtypes: humoral immunity, leading to production of circulating immunoglobulins by B-lymphocytes that bind to pathogens or toxins, and cell-mediated immunity, requiring activated T-lymphocytes to specifically attack pathogens (325).

4.1.1.1.2 The innate immune response

The innate immune system is defined by structures and mechanisms that are encoded in the host's germline and subject to little variation (325). Its receptors recognise shared antigen patterns or toxins and are located on cells that are most likely to encounter pathogens, such as antigen presenting cells (APC) or NK cells. The receptors of the innate immune system target antigens that have very limited variability, i.e. conserved metabolic proteins or carbohydrate structures that are vital to the pathogen itself (320). The generated innate immune response relies on a system of chemokines to control migration of phagocytic cells to affected tissues, e.g. macrophage inflammatory protein 1 (MIP1) that is secreted by epithelial cells. Chemokines also attract lymphocytes into the draining lymph nodes (e.g. RANTES; Regulated on Activation Normal T Cell Expressed and Secreted) to activate an adaptive response (322). Chemokines of the innate immune system are typically secreted locally by APC (mainly dendritic cells and macrophages) and other phagocytic cells but in fulminant inflammatory reactions, chemokines such as those secreted by macrophages (i.e. IL-1, IL-6, IL-12 and TNF-α), can also appear in the bloodstream (322).

4.1.1.1.3 Th1/Th2/Th17 paradigm and formation of an acquired immune response

The acquired immune system is defined by highly specific and variably receptors of T- and B-lymphocytes. Upon activation (binding of presented antigen and costimulatory signals) these lymphocytes undergo clonal expansion and form memory cells to subsequently mount a more targeted immune response in case of re-infection. The type of acquired immune response, humoral (B-lymphocytes) or cell-mediated (T-lymphocytes), is dependent on the preceding innate response (320).

T-helper (Th1 and Th2) cells are T-lymphocytes that help direct the acquired immune response by activation and down regulation of humoral or cellular pathways of immunity, sometimes in feedback loops, such as IL-10 limiting a Th1 response and hence dampening overall inflammation (326). The different cytokines most involved in their activation have traditionally been used to define Th1-type and Th2-type responses; IFN-γ for Th1-type/cell-mediated immunity and IL-4 for Th2-type/humoral immunity (327); but it is important to recognise that these cytokines are also produced by other cell

types and the resulting immune response is directed by combinations of cytokines rather than individual cytokines (328).

An acquired, humoral immune response is best suited to deal with extracellular pathogens and can be triggered by IL-4 secretion by NK cells, or IL-10 secretion by macrophages and monocytes, leading to activation of Th2 helper cells that subsequently produce more IL-4 to stimulate B-cells to differentiate into plasma cells and secrete soluble immunoglobulins (i.e. B-cell receptors). Activated Th2 helper cells can also produce further proinflammatory cytokines such as IL-4, IL-5 or IL-9 as well as anti-inflammatory IL-13.

An acquired, cellular immune response can be triggered by release of IL-12 from macrophages or secretion of IFN-γ from NK cells, leading to activation of Th1 helper cells, that produce more IFN-γ, TNF-α and mount an immune response best suited to deal with intracellular pathogens (322).

Apart from Th1 cells directing cell-mediated immunity, by activation of cytotoxic T-cells and memory T-cells, and Th2 cells directing humoral immunity, by activation of B-cells into plasma cells and formation of memory B-cells, a third T-helper cell population, Th17, has recently been described. Th17 helper cells arise following stimulation with IL-6, IL-23 and transforming growth factor beta (TGF-β) and are inhibited by IL-4 and IFN-γ (329). They play an equally important role to Th1 and Th2 helper cells in bridging the gap between the innate and acquired immune response (212). Th17 helper cells promote inflammation by secretion of IL-17, TNF-α, GMCSF (granulocyote-macrophage colony stimulating factor), KC (keratinocyte chemoattractant, a pro-inflammatory, neutrophil-recruiting chemokine) and IL-6, often leading to excess inflammation and autoimmunity (330). However, early release of IL-17 in infection might also upregulate defensive mechanisms until other T-helper cells are recruited for an acquired immune response, as Th17 pathways are thought to be evolutionary older than the formation of an acquired immune response (331). This early IL-17 response is typically caused by innate release from γδ-T-cells; unusual T-cells that use their T-cell receptor for pattern recognition, thus acting as APC whilst allowing very early clonal expansion at sites of antigen exposure, such as the spleen or mucosa-associated tissues (332).

Another subset of T-helper cells, T-regulatory cells (T_{regs}) arise from a combination of TGF-β and IL-10 (333). An imbalance of T_{regs} and Th17 function is thought to be the underlying mechanism for many autoimmune diseases (330, 331). Accumulation of T_{regs} at the site of infection can sometimes favour pathogen persistence e.g. in leishmaniasis (334) or in *Shistosoma mansoni* infection (335). This mechanism of immune evasion has successfully been targeted in vaccination approaches to bacterial pathogens such as *Mycobacterium bovis*, where knowledge of the regulatory pathways can subsequently guide adjuvant selection and method of vaccine administration (336).

The specific receptors of the acquired immune system only bind to microbial antigens that have been presented to the T-helper cells, which is followed by clonal expansion of specific cytotoxic T-cells mediated by Th1 cells or B-cell proliferation and differentiation (leading to immunoglobulins being produced by plasma cells) mediated by Th2 cells (322). Whilst a Th17-mediated role in protection from infection is undisputed (331), their effects are less well defined and consist of neutrophil-recruiting, enhanced granulopoiesis as well as extramedullary haematopoiesis and a critical role in fighting extracellular pathogens that have escaped Th2 and Th1 effector mechanisms (337).

Immunoglobulins bound to the surface of bacterial pathogens and can activate a cascade of proteins (complement reaction) leading to the elimination of pathogens from the host whereas cytotoxic T-cells may directly attack their target, typically virus-infected cells (322). Subsets of these effector B-

and T-cells form memory cells that can persist for years and induce a longer, more specific (IgG versus IgM immunoglobulins) and more polarised (humoral versus cell-mediated), immune response upon re-encounter with the same antigen (288). A similar effect of polarisation of the immune response may be seen in chronic infection (338) and can be exploited for re-inoculation experiments (339).

4.1.1.2 Detection of the immunological response to bacterial infection

Detection of cytokines to reflect immunological responses is challenging due to their concentrations being low (pg/ml) and the biological substrate (plasma or other intercellular fluids) being highly complex and prone to causing assay interference (340). In the past, some cytokines, such as interferons, were measured in anti-viral assays, where their ability to block viral infection could be quantified in the number of living cells after incubation with virus particles and test substrate. Alternatively, cell migration was observed to assess for chemokines or competitive receptor binding used where capture antibodies were available to detect the molecule of question (341). More recently, ELISA-based immunoassays have been used to measure cytokines in clinical samples but development and validation of ELISA technology can be cumbersome and is not available for cytokines in less researched species, such as non-human, non-lagomorph and non-rodent mammals (342). Detection of messenger RNA (mRNA) by means of reverse-transcriptase quantitative PCR (RT-qPCR) have been employed to measure cytokines at pre-expression level in less researched species such as cats (343). However, depending on the cytokine being measured, these were shown to have poor agreement with detection of the cytokine protein product by ELISA-based techniques (344). Similarly, ELISAs are time-consuming and not suited for detection of cytokines in small sample volumes. Thus these disadvantages of RT-qPCR and ELISA led to the development of bead-based techniques that allow simultaneous detection of multiple cytokines in a high-throughput, flow-cytometry based format that utilise fluorescently labelled beads covered in detection antibodies (340). Performance of bead-based multiplex immunoassays has been comparable to the much slower and substrate-consuming ELISA technology (345), but is still significantly affected by sample handling (i.e. freeze-thaw cycles) and substrate (i.e. serum versus plasma), and these factors need to be evaluated before comparing actual data across studies detecting cytokines from different sources (346).

The cellular immune response to bacterial infection can also be observed and roughly quantified by monitoring haematological parameters (i.e. counts of red and white blood cells) over the course of infection (347). Coagulation parameters, Coombs' tests and osmotic fragility have also been included in routine monitoring of clinical haemoplasmosis in veterinary species (79, 90, 348) as they relate to the intensity of the immune response (349, 350), but may be difficult to perform on the very limited sample volumes that can be obtained from mice.

4.1.2 Previous studies on the immune response to haemoplasma infection in non-rodent species

Protective immunity after primary exposure and recovery from infection has been described in cats for infection with *M. haemofelis* and '*Ca.* M. turicensis' (74, 107).

When assessing protective immunity to re-infection with *M. haemofelis*, using RT-qPCR to feline cytokine mRNA, Hicks, Willi (107) did not find a clearly defined Th1/Th2 or Th17 differentiation between cats undergoing primary *M. haemofelis* infection and cats recovered from *M. haemofelis* infection after re-inoculation and re-infection. There was an increase in IL-10 mRNA (representing an anti-inflammatory, Th2 response) during peak bacteraemia in naïve infection but not in the re-infection group, as well as a potential TNF-α mRNA and IL-6 mRNA increase in the re-infection group

that was not present in the naïve group, which was interpreted as a potential Th17 response, but this could not be confirmed with the employed methodology.

In a similar study, assessing the occurrence of protective immunity and feline cytokine response to '*Ca.* M. turicensis' infection using RT-qPCR to feline cytokine mRNA, Novacco, Boretti (124) found an early IFN-γ mRNA (representing a Th1 response) increase in naïvely infected but not in re-inoculated re-infection cats, and an increase in IL-4 mRNA (representing a Th2 response) in re-infection cats as well as during the later stages of naïve infection. The authors concluded that Th1 responses play a role in protective immunity, but it must be noted that the infection kinetics investigated were shortlived ('*Ca.* M. turicensis' being undetectable after less than three months whilst *M. haemofelis* infection typically still shows a lasting plateau-phase at this point) and clinical changes were very mild following '*Ca.* M. turicensis' naïve infection when compared to *M. haemofelis*. In line with the lack of a Th2 humoral response shown in the Novacco, Boretti (124) study with '*Ca.* M. turicensis', another experiment showed a similar lack of humoral immunity in protection against *M. haemofelis* infection, when the transfusion of feline convalescent plasma from cats previously recovered from *M. haemofelis* infection to naïve cats did not prevent subsequent *M. haemofelis* infection (138).

Earlier studies demonstrated an acute phase response in cats following infection with *M. haemofelis* and '*Ca.* M. haemominutum' (108). A humoral immune response to a *M. haemofelis* heat-shock protein (DNAK) was demonstrated following *M. haemofelis* and '*Ca.* M. turicensis' infection in cats (94, 351) but was not associated with protective immunity to either of these feline haemoplasmas.

Studies on the porcine haemoplasma *M. suis* have concentrated on haematological outcomes of infection, such as anaemia (111), and documenting the humoral immune response to *M. suis* antigens such as the *M. suis* heat-shock protein HSP1 in search of vaccine candidates (142). Inoculation of HSP1 into naïve pigs was found to be immunogenic (91) but was not protective from subsequent *M. suis* infection (120).

Other veterinary species and their immune response to haemoplasma infection have not yet been examined at molecular level.

4.1.3 Previous studies on haemoplasmosis in rodents and the murine immune response to haemoplasma infection

Anaemia (more specifically, approximately a 50% decrease in both red cell counts and haemoglobin concentrations) secondary to *M. haemomuris* (then named *Bartonella muris*) infection has been observed previously in young rats, rabbits, guinea pigs and mice, although no molecular characterisation of the used haemoplasma isolate could be attempted at the time to confirm its phylogeny (240). The authors also observed complement fixation at high dilutions of serum from infected rabbits when using bacterial haemoplasma suspensions from putative blood cultures in leptospira medium as antigen, suggesting that a Th2 humoral immune response was present (240). Complement binding was later also observed in infected mice (194).

As has been observed in cats, pigs and other host species (see 1.2) infected with haemoplasmas, splenomegaly and peracute death have also been observed in *M. haemomuris* infection in rats (250) and mice (197). The study by Rikihisa, Kawahara (197) also describes probing of crude *M. haemomuris* antigen preparations with convalescent mouse sera by Western immunoblot, and found five major immunoreactive antigens ranging in size from 45 to 118kDa (Rikihisa, Kawahara et al. 1997). Identification of the antigens was not possible due to the whole genome sequence of *M. haemomuris* not being available to identify isolated proteins by mass spectroscopy. Similarly, as

there is a lack of an *in vitro* cultivation system, obtaining higher *M. haemomuris* protein quantities for purification of antigens was not feasible from mice.

In mice, acute infection with *E. coccoides* appeared to have an interferon-lowering effect during ongoing viral infection experiments, potentially via interference with a Th1 immune response (253). The presence of a humoral immune response was confirmed by blocking infectivity of inocula by prior incubation with convalescent sera before inoculation via injection into susceptible mice (253). Iralu and Ganong (255) demonstrated hemagglutination of washed normal red blood cells of mice when incubated with washed *E. coccoides* infected red blood cells and concluded that infection occurs independently of humoral elements as they were not added to the *in vitro* experiment.

4.1.4 Principal component analysis in immunology

Immunological data are often analysed with simple tests, even when multiple relationships between the variables are possible or expected (352). These analyses often limit the statistical methods to non-parametric tests, as immunological data are rarely normally distributed between individuals (352). Often, there is a common underlying biological relationship to the observed changes that is hidden from direct observation of the data, such as 'down-regulation' or a 'shift' in immune response (353). Such relationships might also be subject to change depending on other variables, e.g. one cytokine might drive different effects in different environments, and the lack of knowledge about possible relationships in pilot studies must be accounted for (353). Multivariate analyses can account for such relationships amongst the variables if certain assumptions on the dataset, such as normality and homogeneity of variances, are met by the data. Principal component analysis (PCA) does not require such assumptions (354) and thus is better suited for use on highly structured data than multivariate analyses.

Immunological studies often aim to identify clusters of immunological changes (profiles) based on previously described immunological changes, unless more specific questions on risk factors or known causative relationships can be asked (353). In the mouse model of haemoplasma infection, the desired clusters of immunological changes would ideally differentiate mice that show protection from re-infection with *M. haemomuris* from mice undergoing naïve *M. haemomuris* infection, despite the absence of other defined variables such as degree of anaemia, splenomegaly or possible other, as yet uncharacterised, laboratory changes associated with haemoplasma infection in mice in our model. Only when immunological changes reflecting protection from infection or haematological/clinical changes are better defined in the mouse model, can the model be used to identify risk factors for more severe clinical disease. Such knowledge of the immunological changes can also help investigate further research questions on transmission, therapy or vaccination against *M. haemomuris* infection.

Principal component analysis identifies and investigates clusters within a dataset of correlated variables (such as cytokines) and subsequently guides further analyses without making assumptions about the underlying dataset (353). Principal component analysis reduces the dimensionality of the data by creating summary variables reflecting changes in two or more of the original variables. These summary variables are referred to as principal components or factors, depending on the conceptual assumptions made. The term factor is used when assuming that an underlying force (not included in the measurements) is driving the observed changes and associations. The term component reflects intrinsic relationships within the dataset of correlated variables that can be reduced in dimensions for a simpler form of analysis. Principal components are linear combinations of the underlying variables, although non-linear relationships may still be accounted for by transformation of the dataset (355). Each original datapoint is related to the new principal component by its 'loading',

reflecting the weight the original variable has on the extracted component; bigger loadings reflecting more influential variables. Loadings allow each datapoint to be assigned a new 'component score', that represents the original datapoint on the new component axis and can subsequently be used for analyses without reducing the information content (353). The clustering of component scores (e.g. reflecting cytokine concentrations on the extracted principal component) in reduced dimensions (e.g. data can now be plotted on three component axes instead of 30 cytokines by having all 'high' cytokines loading on a single component) and their relationship with other variables of infection can then be used to allow the dataset to better reflect the underlying immune response e.g. showing a Th1/Th2 bias (356).

4.1.5 What does it take to validate mouse models?

A validated mouse model is one in which there is significant overlap and analogies of the factors contributing to the model and the modelled disease (218). However, significance is a subjective measure and defined by a researcher and acceptance through the scientific community. However, it is generally assumed that there must be a large amount of overlapping data e.g. in response to infection, in the underlying biological phenomenon or the phylogenetic characteristics of a pathogen or model species. The data required for this overlap are directly dependent on the research objective, and some models might be better suited than others depending on the research objective (357).

Research objectives must be clearly articulated to choose which animal model would be most suitable, or whether a single animal model or a combination or models would be more suitable for the infectious disease research (357). Different objectives e.g. investigating pathophysiology, or virulence factors versus the host immune response to infection, may require different animal models. Colby, Quenee (357) list several viral and bacterial infections which have mouse, guinea pig, ferret or non-human primate models available, each having different advantages depending on the research objective being investigated. For most measures to prevent infection with viral or bacterial pathogens, preference has been given to mouse models due to their small size allowing cost-effective studies with relatively high animal numbers, even if they require a mouse-adapted strain of pathogen to be used and only few or no clinical signs are seen in mouse models e.g. for Ebola or Influenza virus (357).

To select and validate the best model for a given research objective, Denayer, Stöhr (313) proposed a fit-for-purpose validation of animal models according to number of different criteria:

- face validity, the similarity in the biology and symptoms between the model and disease
- predictive validity, the similarity of clinical interventions (e.g. drugs) leading to similar effects
- target validity, the similarity of the role of the target of investigation (e.g. receptor) in the model and disease.

Although these criteria can be employed for general validation of an animal model, fit-for-purpose weighting of these criteria is suggested for individual objectives e.g. when studying pathological mechanisms of a disease, the face validity of the model is more important than other criteria.

Using a similar but more detailed scoring system, Sams-Dodd (358) concluded that a model's face validity is higher when more symptoms match. For our current surrogate mouse model of haemoplasma infection that has been established in Chapter 3, more clinical, pathological and laboratory data need to be collected to fully describe the biology and symptoms for face validity, which is difficult due to mice often hiding clinical signs (312) and their small size limiting collection of multiple blood samples over the course of *M. haemomuris* infection from the same animal. Scores of

model face validity (358) are also based on the phylogenetic relationship between the host species of model and disease, but are only established for human disease models. In human disease models a higher score is assigned to primates compared to other less related species for example, but this cannot be extrapolated to other veterinary species as they show a more distant phylogenetic relationship to mice than humans do (359). Ultimately, an animal model should only be used when the research question cannot be answered by other non-experimental animal means, typically shifting the use of models to pre-clinical drug testing and vaccine development, where repeatability cannot easily be achieved in human cohorts (360). However, as haemoplasmas remain unculturable *in vitro*, even basic questions regarding their pathogenic mechanisms cannot be studied outside of the host, thus adding an additional criterion of being able to investigate basic pathophysiology of haemoplasmosis to a fit-for-purpose definition of mouse model validation.

The above considerations on model development and validation suggest that the current mouse model of haemoplasma infection described in Chapter 3 appears to lack a proof of repeatability due to the low mouse numbers used (group sizes of 8 mice to study infection kinetics after primary inoculation, cross-inoculation and protection from re-infection) and the high proportion of infection failures (4 out of 8 mice during primary inoculation) observed. However, protection from re-infection with *M. haemomuris* was confirmed in mice, hence allowing us to use this mouse model to assess the immunological response to *M. haemomuris* infection and protection from-re-infection. Other research objectives to describe the pathophysiology of different haemoplasmas cannot be studied in our mouse model. There are no data currently available listing variables that are affected by haemoplasma infection yet alone determining whether *M. haemomuris* infection causes clinical signs of haemoplasmosis and associated laboratory changes in C57BL/6 mice.

4.1.6 Objective

This chapter explores the effect of *M. haemomuris*-infection and re-infection on murine haematology parameters, spleen size and plasma cytokine expression over two weeks following inoculation with *M. haemomuris*. Our aim was to define the pathological and laboratory changes associated with haemoplasmosis due to *M. haemomuris* in mice and use PCA to identify a cytokine signature of protection from re-infection compared to naïve infection with *M. haemomuris*. These studies will inform further studies that could provide possible insights into the immunisation against haemoplasma infection in other species, using *M. haemomuris* as a surrogate pathogen.

4.2 MATERIALS AND METHODS

4.2.1 Animals

All mice were female, SPF C57BL/6 and sourced from Harlan Laboratories UK Ltd., Loughborough, UK. The mice used for the studies described in this chapter were split into 'haematology study' and 'cytokine expression study' animals due to limitations on analyses possible on each blood sample taken due to sample volume requirements and processing time. Both the haematology study and the cytokine expression study included samples collected over the first 14 days of naïve *M. haemomuris* infection, and the cytokine expression study also included samples collected over the first 14 days after re-inoculation with *M. haemomuris*.

4.2.1.1 Ethical approval

All studies involving laboratory rodents were subject to ethical review (Animal Welfare Ethical Review Body of the University of Bristol) and covered by a Home Office licence (PPL PCFCBE2EB).

4.2.1.2 Housing conditions

All mice were housed and monitored as previously described in 2.2.3.2.

4.2.1.3 Screening mice for pre-existing M. haemomuris-infection

Upon arrival at the facility, mice were allowed an acclimatisation period and one week before all experiments, mice were uniquely identified and had a 10µl blood sample withdrawn for generic haemoplasma qPCR analysis to screen for pre-existing *M. haemomuris* infection, as outlined in 2.2.3.3.

4.2.1.4 Haemoplasma inoculation

4.2.1.4.1 Cryopreservation

The cryopreservation of inocula for infecting mice was modified from previous studies (2.2.3.7) to account for potential DMSO toxicity. In brief, blood samples for cryopreservation were collected as previously described but mixed with 20% (v/v) glycerol (Sigma) instead of DMSO, and frozen at -80°C until use where cryopreservation was required. When uniform inocula needed to be generated to infect large groups of mice, blood was collected and used immediately, without cryopreservation, to try and allow for optimal *M. haemomuris* viability.

4.2.1.4.2 Inoculation Procedure

The inoculation procedures for the studies described in this chapter were as for the procedures described in 2.2.3.4.1, other than the handling of mice prior to inoculation was different as it required optimisation to accommodate the increased mouse number as outlined below in 4.2.1.4.2.1.

4.2.1.4.2.1 High-throughput inoculation

Inoculation procedures were modified for use in the studies described in this chapter so that they could be completed by one person within a period of five minutes of obtaining the infected blood sample inoculum from an amplifier mouse. To minimise the time spent handling and identifying the mice, subsets of the experimental mice were separated into small plastic containers (containing litter and a cereal treat) prior to inoculation and placed back into their original cages upon completion of inoculation (**Figure 25**).

Figure 25 *Cages containing a subset of mice for the cytokine expression study, taken out and placed on the work surface before transferring the individual mice into the small plastic containers (pipette tip containers, shown with open lids, containing litter and a cereal treat) for easy and quick inoculation. Separating the mice using these pipette containers avoided time delays following inoculum collection and inoculation, as the mice could be identified ahead of inoculum collection. Mice were removed from the small plastic containers, inoculated with 0.1ml Mycoplasma haemomuris-containing mouse blood inoculum intraperitoneally and placed back into their respective cages. Inoculation of all mice receiving the same inoculum could be completed in less than five minutes by a single researcher.*

4.2.1.5 Haematology study

4.2.1.5.1 Inoculation schematic

An overview of the inoculation schematic for the haematology study is given in **Figure 26**.

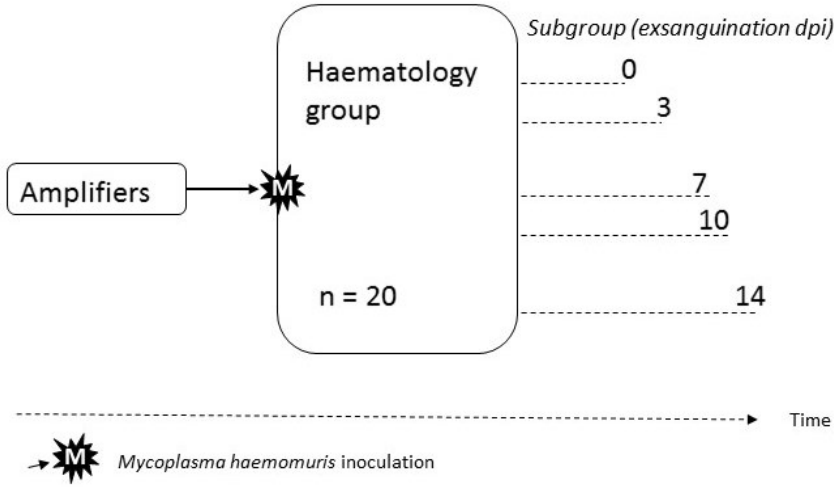


Figure 26 *Inoculation schematic to outline the experimental design for the haematology study. Sixteen mice were inoculated with freshly collected Mycoplasma haemomuris-containing mouse blood and four mice each were exsanguinated on days 3, 7, 10 and 14 post inoculation. Four mice, housed under identical conditions, were not inoculated but exsanguinated on day 0, the day of inoculation of the other groups, as controls. Dpi: days post inoculation.*

Twenty mice were split into five groups of four mice and each group housed in the same cage. Randomisation was achieved at the level of mouse delivery from the supplier and information regarding the original litters the mice were from was not available to the researcher.

At the start of the experiment, 16 mice were inoculated with *M. haemomuris* using inocula generated from freshly collected blood from amplifier mice. The four remaining mice were not inoculated and were exsanguinated under terminal anaesthesia as previously described (2.2.3.7) on day 0 of the experiment to act as uninfected controls. Further subsets of 4 mice each were exsanguinated on 3, 7, 10 and 14 days post-inoculation (dpi). Anticoagulated blood collected at the time of exsanguination (see 4.2.2.1.3) underwent haematological analysis, as described below in 4.2.4.

4.2.1.5.2 Generating inocula in amplifier mice

The haematology study was preceded by production of amplifier mice to generate the inocula for use in the haematology group (**Figure 26**). The amplifier mice preceding the haematology study were produced by the inoculation of a *M. haemomuris* isolate that had undergone seven serial passages in C57BL/6 mice since sourcing the original rodent haemoplasma isolates from field mice (2.2.1).

4.2.1.5.2.1 Description of inocula used to inoculate the amplifier mice preceding the haematology study

Three ten-week-old mice were inoculated with *M. haemomuris*-infected blood immediately following its collection from the tail vein of a *M. haemomuris*-infected mouse as previously described 2.2.3.4.1. The origin and composition of inocula used to inoculate the laboratory amplifier mice is given in **Table 27**.

Mouse ID	Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	*M. haemomuris* dose in 0.1ml of inoculum per mouse
Amplifier H1, Amplifier H2 and Amplifier H3	*M. haemomuris*	Blood from a C57BL/6 mouse	None	1:4	6.91E+04

Table 27 *Origin and composition of inocula used to inoculate the amplifier mice preceding inoculation of the haematology group. These amplifier mice were subsequently used to generate inocula for the experiment as described in* ***Figure 26****. Inoculum haemoplasma doses were calculated as in2.2.1.2.2.2. PBS, phosphate buffered saline.*

Following inoculation, amplifier mice were monitored for *M. haemomuris* infection by taking blood samples of 10µl for qPCR analysis, using a *M. haemomuris*-specific qPCR assay, as described previously (3.2.2.3.2). At seven dpi (when harbouring 1.23E+07 *M. haemomuris* genome copies per µl blood), one mouse ('Amplifier H1', see **Table 27**) was exsanguinated under terminal anaesthesia, as previously described (2.2.3.7), to produce the inocula for the haematology group mice. The remaining two amplifier mice ('Amplifier H2' and 'Amplifier H3') remained unused but had been generated for contingency considerations (i.e. if less than the required blood volume could be collected from one or two of the amplifier mice) and were included in subsequent experiments for *in vitro* culture studies, as described in Chapter 5.

4.2.1.5.3 Monitoring of haematological changes during primary *M. haemomuris* infection

4.2.1.5.3.1 Description of inocula used for the haematology group

Twenty ten-week-old mice were housed in five cages (A, B, C, D and E) of four mice each. After an acclimatisation period of two weeks, sixteen mice in cages B to E were each inoculated with 0.1ml of a *M. haemomuris*-containing inoculum that had been prepared from freshly collected, heparinised blood, generated in amplifier mice. The origin and composition of the inocula are given in **Table 28**.

The sixteen mice were inoculated in sequential order of mouse '1' to '4' from cages B to E using the technique described in 2.2.3.4.1. The four mice in cage "A" did not receive any inocula and acted as controls as described in 4.2.1.5.1.

Mouse ID	Cage	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	*M. haemomuris* dose in 0.1 ml of inoculum per mouse	Subgroup (refers to exsanguination dpi)
1, 2, 3, 4	A	n/a	n/a	n/a	n/a	0
1, 2, 3, 4	B	Amplifier H1	None	1:4	3.08E+08	3
1, 2, 3, 4	C	Amplifier H1	None	1:4	3.08E+08	7
1, 2, 3, 4	D	Amplifier H1	None	1:4	3.08E+08	10
1, 2, 3, 4	E	Amplifier H1	None	1:4	3.08E+08	14

Table 28 *Origin and composition of inocula used to inoculate the haematology group mice. One amplifier mouse (**Table 1**) was killed at seven days post inoculation (dpi) and inocula generated from its blood used without cryopreservation. On day 0 of the experiment, all mice in cages B to E were inoculated with M. haemomuris-infected blood. Cage A mice were not inoculated and acted as controls. The mice in subgroup 0 were subsequently exsanguinated for collection of EDTA-anticoagulated blood, followed by the same exsanguination procedure for each subgroup of four mice on 3, 7, 10 and 14 dpi. PBS, phosphate buffered saline. n/a, not applicable.*

Following inoculation, mice were then exsanguinated in groups of 4 mice per day, at 0 (without prior inoculation) 3, 7, 10 and 14 dpi to collect EDTA-anticoagulated blood for haematological analysis.

4.2.1.5.3.2 Monitoring of haemoplasma infection

As repeated blood sampling may have interfered with haematological parameters, no daily sampling to monitor for *M. haemomuris* infection was attempted from the haematology group mice. Samples to determine *M. haemomuris* loads via qPCR were taken at post-mortem examination as will be described later in 4.2.2.1.2.

4.2.1.6 Cytokine expression study

4.2.1.6.1 Inoculation schematic

An overview of the inoculation schematic for the cytokine expression study is given in **Figure 27**.

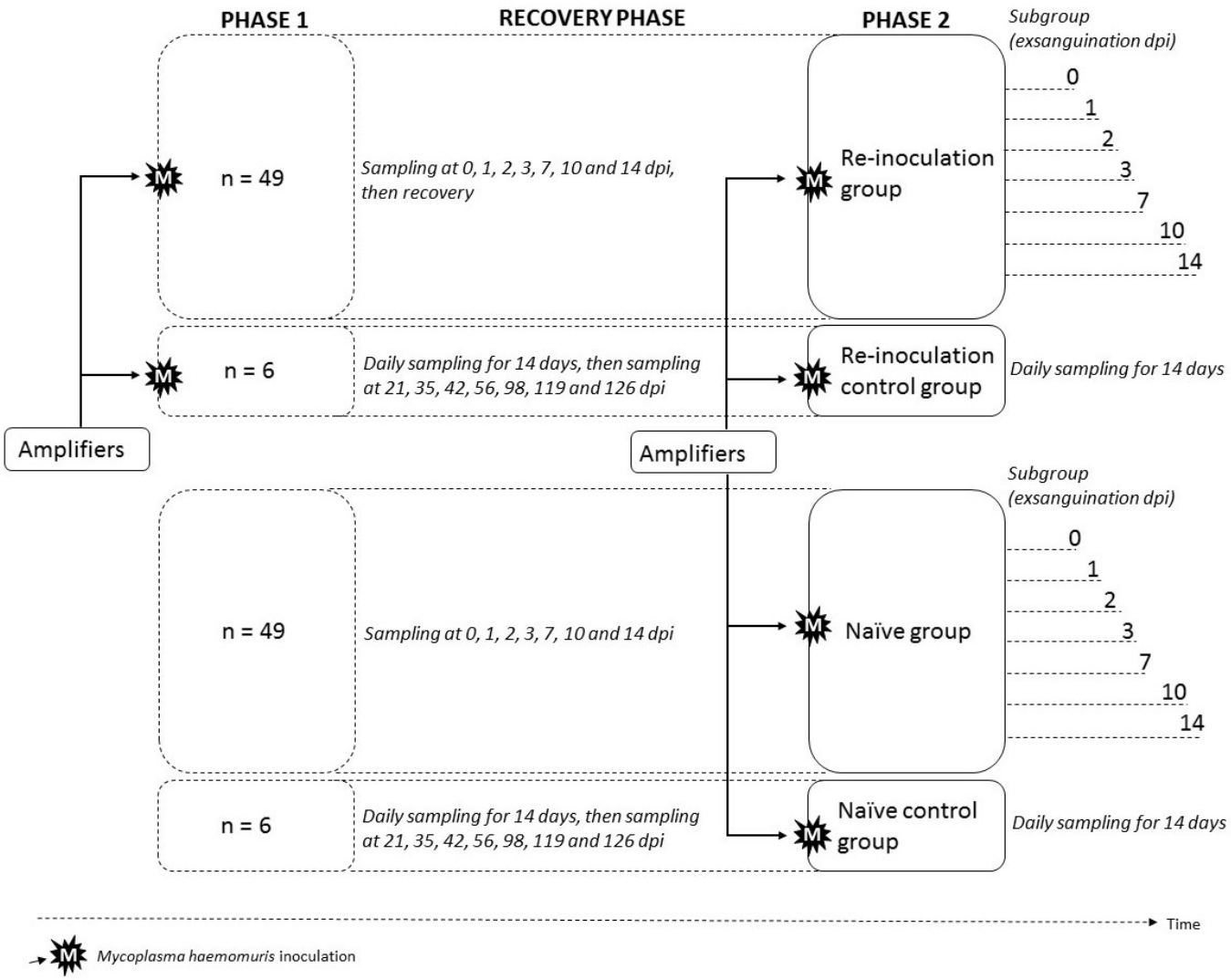


Figure 27 *Inoculation schematic to outline the experimental design of the cytokine expression study. In Phase 1 of the experiment, half of the mice (re-inoculation group and re-inoculation control group) were inoculated with M. haemomuris and subsequently monitored by qPCR for M. haemomuris genome copies per µl blood. The other half of the mice (naïve group and naïve control group) remained naïve to infection but was subjected to the same procedures (blood sampling: naïve group mice were sampled as the re-inoculation group mice and the naïve control group mice were sampled as the re-inoculation control group mice) and housed under the same conditions as the mice inoculated with M. haemomuris. Control groups (re-inoculation control group and naïve control group) were used to allow more frequent blood sampling following inoculation without potentially interfering with cytokine expression in the main experimental groups that subsequently were not sampled following Phase 2 inoculations. Following recovery from infection of the inoculated mice in the Recovery phase, all mice were inoculated with M. haemomuris-containing blood In Phase 2 of the experiment. Naïve group mice and re-inoculation group mice were exsanguinated in subgroups of seven mice on days 0, 1, 2, 3, 7, 10 and 14 post Phase 2 inoculations with M. haemomuris. Groups exsanguinated on 0 days post inoculation (dpi) were not inoculated in Phase 2 before exsanguination for sample collection. Control mice (naïve control group and re-inoculation control group) were used to monitor naïve infection and re-infection following Phase 2 inoculations for 14 days as no consecutive sampling was attempted in the main experimental mice (naïve group and re-inoculation group). Two sets of amplifier mice (one for Phase 1 and one for Phase 2) were used to generate inocula for all groups. Blood was pooled from two amplifier mice each to generate one inoculum in order to ensure comparable doses of M. haemomuris copies per mouse to be inoculated. Inoculum compositions are given in* ***Table 30*** *and* ***Table 32****.*

Ninety-eight experimental mice were split into two groups of 49 mice each. These groups comprised seven subgroups of seven mice each and were housed in groups of four and three mice per cage as summarised later in **Table 33**. Randomisation was achieved at the level of mouse delivery from the supplier and information regarding the original litters the mice were in was not available to the researcher. Experimental groups were matched in age to address potential effects of ageing on cytokine expression and immunity. One group (naïve group) of 49 mice was inoculated with *M. haemomuris* to assess plasma cytokine expression in response to primary *M. haemomuris* infection and another one group (re-inoculation group) was inoculated with *M. haemomuris*, recovered from infection and re-inoculated with *M. haemomuris* to assess plasma cytokine expression associated with partial protection from re-infection as was described in the infection studies in Chapter 3. Frequent serial blood sampling of the 98 main experimental mice would likely have interfered with plasma cytokine expression, and so could not be performed, but daily monitoring of *M. haemomuris* genome copies per µl blood was required for confirmation of inoculum viability, to monitor recovery from infection and assessment of protective immunity. To resolve the problem of being unable to serially sample the mice to monitor *M. haemomuris* infection, two groups each of six control mice were inoculated using the same inoculum as the main experimental mice to be used for frequent monitoring of *M. haemomuris* genome copies within their blood; the naïve control group was inoculated the same as was the naïve group and the re-inoculation control group was inoculated the same as was the re-inoculation group. Two sets of amplifier animals were used to produce inocula with viable *M. haemomuris* preceding Phase 1 and Phase 2 of the experiment.

Phase 1 of the experiment (see **Figure 27**) represents primary infection with *M. haemomuris* of the re-inoculation group and inoculation of the re-inoculation control group. Phase 1 also includes the start of the experiment for the naïve group and naïve control group. Infection with *M. haemomuris* was monitored at 0, 1, 2, 3, 7, 10 and 14 dpi in the re-inoculation group (samples from the naïve group were taken on the same days but did not undergo further testing) and daily for 14 dpi in the re-inoculation control group (samples from the naïve control group were taken on the same days but did not undergo further testing).

The 'Recovery phase' between Phase 1 and Phase 2 represents the period when mice, previously inoculated with *M. haemomuris* 'recovered' from *M. haemomuris* infection. Recovery was monitored in the re-inoculation control group to avoid sampling of the re-inoculation group. The naïve control group was subject to the same blood sampling procedures as the re-inoculation control group, but samples did not undergo further testing. No samples were taken from the naïve group and the re-inoculation group during the Recovery phase.

In Phase 2 of the experiment, the re-inoculation group (except subgroup 0) and re-inoculation control group were re-inoculated with *M. haemomuris.* Phase 1 also included the primary *M. haemomuris* inoculation of the naïve group mice (except subgroup 0) and naïve control group. Following Phase 2 inoculations, naïve group mice and re-inoculation group mice were exsanguinated in sets of seven mice per day and group on days 0, 1, 2, 3, 7, 10 and 14 dpi to collect EDTA-plasma for cytokine analysis. Subgroup names (see **Figure 27**) refer to the dpi of exsanguination. Naïve controls and re-inoculation controls were monitored for *M. haemomuris* infection for 14 dpi.

4.2.1.6.2 Generating inocula in amplifier mice

The experiment was split into Phase 1 and Phase 2 and each phase was preceded by production of amplifier mice to generate inocula for use in the two main experimental groups and two control groups (**Figure 27**). The amplifier mice preceding Phase 1 of the experiment were produced by the inoculation of isolates that had undergone seven serial passages in C57BL/6 mice since sourcing the original rodent haemoplasma isolates from field mice (*Apodemus* spp.) samples. The amplifier mice preceding Phase 2 of the experiment were produced by the inoculation of isolates that had undergone eight serial passages in C57BL/6 mice since isolation from field mice (2.2.1).

4.2.1.6.2.1 Description of inocula used for the amplifier mice preceding Phase 1

Eight nine-week-old mice were inoculated with 0.1 ml cryopreserved blood containing *M. haemomuris.* Origin and composition of inocula used to inoculate these laboratory mice is given in **Table 29**.

Mouse ID	Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	*M. haemomuris* dose in 0.1 ml of inoculum per mouse
Amplifier C1, Amplifier C2, Amplifier C3, Amplifier C4, Amplifier C5, Amplifier C6, Amplifier C7 and Amplifier C8	*M. haemomuris*	Blood from C57BL/6 mouse	20% v/v Glycerol	None	2.63E+06

Table 29 *Origin and composition of inocula used to inoculate the amplifier mice preceding Phase 1 of the cytokine expression study. These mice were subsequently used to generate further inocula for the experiment as illustrated in* ***Figure 27****. The donor mouse to generate inocula used in amplifier mice preceding Phase 1 had been exsanguinated by cardiac puncture at 14 dpi with M. haemomuris; eight samples were cryopreserved at – 80°C and stored for 28 days prior to use. M. haemomuris dose was calculated as in 2.2.1.2.2.2. PBS, phosphate buffered saline.*

Inocula were allowed to defrost at room temperature for 10 minutes and were inoculated intraperitoneally as previously described (2.2.3.4.1). Following inoculation, the eight amplifier mice preceding Phase 1 were monitored daily for *M. haemomuris* infection using a species-specific *M. haemomuris* qPCR assay as previously described (3.2.2.3.2).

4.2.1.6.2.2 Inocula generated from amplifier mice preceding Phase 1

Seven dpi sets of two amplifier mice each were exsanguinated through cardiac puncture and heparinised whole blood was gently mixed in a 5ml Sterilin tube (Thermo Fisher) to produce an inoculum aliquot (**Table 30**) to inoculate the re-inoculation group and re-inoculation control group in Phase 1 of the cytokine expression study (see **Figure 27**). Pooling of blood from two amplifier mice each was required to ensure volumes large enough to inoculate multiple subgroups of the main experimental mice from the same inoculum aliquot. The inocula for Phase 1 were used within ten minutes of exsanguinating the amplifier mice and did not undergo further storage or preservation. The remaining four amplifier mice (‘Amplifier C5’ to ‘Amplifier C8’) remained unused but had been generated for contingency considerations (i.e. if less than the required blood volume could be collected from one or two of the amplifier mice) and were included in subsequent experiments for *in vitro* culture studies, as described in Chapter 5.

Inoculum aliquot ID	Origin	Blood volume obtained from exsanguination (ml)	*M. haemomuris* copies per µl blood	Dilution ratio of inoculum in PBS
Pool 1	Amplifier C1	0.6	6.02E+04	1:4
	Amplifier C2	0.6	3.04E+04	
Pool 2	Amplifier C3	0.5	7.91E+05	1:4
	Amplifier C4	0.5	7.54E+04	

Table 30 *Origin and composition for Phase 1 inoculum aliquots that were used to inoculate the re-inoculation group and re-inoculation control group in Phase 1 of the cytokine expression study (**Figure 27**). M. haemomuris copies per µl blood were calculated as in 2.2.1.2.2.2. PBS, phosphate buffered saline.*

4.2.1.6.2.3 Description of inocula used for the amplifier mice preceding Phase 2

Eighteen weeks after the Phase 1 inoculations, twelve nine-week old mice were inoculated with blood containing *M. haemomuris.* The origin and composition of inocula used to inoculate these laboratory mice is given in **Table 31**.

Mouse ID	Organism	Origin	Cryopreservation	Dilution ratio of inoculum in PBS	*M. haemomuris* dose in 0.1ml of inoculum per mouse
Amplifier C9 Amplifier C10 Amplifier C11 Amplifier C12 Amplifier C13 Amplifier C14 Amplifier C15 Amplifier C16 Amplifier C17 Amplifier C18 Amplifier C19 Amplifier C20 and Amplifier C21	*M. haemomuris*	Blood from C57BL/6 mouse	None	1:4	5.21E+06

Table 31 *Origin and composition of inocula used to inoculate the amplifier mice preceding Phase 2 of the cytokine expression study. These mice were subsequently used to generate further inocula for the experiment as illustrated in **Figure 27**. The donor mouse to generate inocula used in amplifier mice preceding Phase 2 had been exsanguinated by cardiac puncture at 15 dpi with M. haemomuris and blood was used without further cryopreservation. M. haemomuris dose was calculated as in 2.2.1.2.2.2. PBS, phosphate buffered saline.*

Inocula were used without cryopreservation and inoculated intraperitoneally as previously described (2.2.3.4.1) within ten minutes of exsanguination of the donor mouse, used to collect *M. haemomuris*-infected blood. Following inoculation, the 12 amplifier mice preceding Phase 2, were monitored daily for *M. haemomuris* infection using a species-specific *M. haemomuris* qPCR assay as previously described (3.2.2.3.2).

4.2.1.6.2.4 Inocula generated from amplifier mice preceding Phase 2

Six days after inoculation, subsets of two amplifier mice each were exsanguinated through cardiac puncture and heparinised whole blood was gently mixed in a 5ml Sterilin tube (Thermo Fisher) to produce an inoculum aliquot (**Table 32**) to inoculate re-inoculation group, naïve group, re-inoculation control group and naïve control group in Phase 2 of the cytokine expression study (see **Figure 27**). Pooling of blood was required to ensure volumes large enough to inoculate multiple subgroups of the main experimental mice from the same inoculum aliquot. Six amplifier mice remained unused due to contingency considerations (i.e. collecting less than the required blood volume from one or more mice) and were included in subsequent experiments in Chapter 5.

Inoculum aliquot	Origin	Blood volume obtained from exsanguination (ml)	*M. haemomuris* copies per µl blood	Dilution ratio of inoculum in PBS
Pool 3	Amplifier C9	0.4	5.65E+07	1:4
	Amplifier C10	0.6	5.50E+07	
Pool 4	Amplifier C11	0.5	4.86E+07	1:4
	Amplifier C12	0.4	3.43E+07	
Pool 5	Amplifier C13	0.5	2.91E+07	1:4
	Amplifier C14	0.6	5.53E+07	

Table 32 *Origin and composition for Phase 2 inoculum aliquots that were used to inoculate the re-inoculation group, naïve group, re-inoculation control group and naïve control group in Phase 2 of the cytokine expression study (**Figure 27**). M. haemomuris copies per µl blood were calculated as in 2.2.1.2.2.2. PBS, phosphate buffered saline.*

4.2.1.6.3 Phase 1: Primary infection of re-inoculation group and re-inoculation controls

4.2.1.6.3.1 Re-inoculation group: Description of inocula used for primary infection

Forty-nine, nine-week-old mice were split into seven subgroups of seven mice each and housed in cages of four and three animals per subgroup. Mice '1' to '4' were housed in the first cage and mice '4' to '7' were housed in the second cage of each group (see cage allocations in **Table 33**). After an acclimatisation period of two weeks, mice were inoculated with 0.1ml of a *M. haemomuris*-containing inoculum that had been generated in amplifier mice preceding Phase 1 of the experiment and prepared in aliquots by pooling freshly collected, heparinised blood and diluting it with PBS as described in 4.2.1.6.2.2. Origin and composition of inoculum aliquots has been given in **Table 30** above. Two inoculum aliquots were used as inocula for seven experimental groups of seven mice each. The origin (inoculation aliquot) and composition of the inocula used to inoculate the re-inoculation group in Phase 1 is given in **Table 33**.

Group inoculated	Mouse ID	Cage ID	*M. haemomuris* dose in 0.1ml of inoculum per mouse	Origin (inoculum aliquot)	Subgroup (exsanguination dpi)
			Phase 1		
Re-inoculation group	1, 2, 3, 4	O	1.13E+07	Pool 1	0
	5, 6, 7	P			
	1, 2, 3, 4	Q	1.13E+07	Pool 1	1
	5, 6, 7	R			
	1, 2, 3, 4	S	1.13E+07	Pool 1	2
	5, 6, 7	T			
	1, 2, 3, 4	U	1.13E+07	Pool 1	3
	5, 6, 7	V			
	1, 2, 3, 4	W	1.93E+07	Pool 2	7
	5, 6, 7	X			
	1, 2, 3, 4	Y	1.93E+07	Pool 2	10
	5, 6, 7	Z			
	1, 2, 3, 4	A2	1.93E+07	Pool 2	14
	5, 6, 7	B2			
			Phase 1		
Naïve group	1, 2, 3, 4	A	n/a	n/a	0
	5, 6, 7	B			
	1, 2, 3, 4	C	n/a	n/a	1
	5, 6, 7	D			
	1, 2, 3, 4	E	n/a	n/a	2
	5, 6, 7	F			
	1, 2, 3, 4	G	n/a	n/a	3
	5, 6, 7	H			
	1, 2, 3, 4	I	n/a	n/a	7
	5, 6, 7	J			
	1, 2, 3, 4	K	n/a	n/a	10
	5, 6, 7	L			
	1, 2, 3, 4	M	n/a	n/a	14
	5, 6, 7	N			

Table 33 *Origin and composition of inocula used to inoculate the mice in Phase 1 of the cytokine expression study. On day 0 of Phase 1 the 49 re-inoculation group mice were inoculated with 0.1ml of an inoculum that had been produced in aliquots (**Table 30**) by pooling and diluting blood from amplifier mice (**Table 29**). Phase 1 represents the primary M. haemomuris infection of the re-inoculation group and Phase 2 represents the re-inoculation of the re-inoculation group and coinciding primary infection of the naïve group. No mice were exsanguinated during Phase 1. Dpi, days post (Phase 2) inoculation. N/a, not applicable.*

Mice were inoculated in sequential order of mouse '1' to '7' per subgroup and subgroups in the order of subgroups '0' to '14' using the technique described in 4.2.1.4.2. To ensure inoculum composition did not change within the same subgroup, the remaining inoculum aliquot was discarded if its volume dropped below 0.8ml. Exsanguination of amplifier mice to generate an inoculation aliquot and inoculation of the same aliquot into experimental mice was completed within ten minutes.

4.2.1.6.3.2 Re-inoculation control group: Description of inocula used for primary infection

After inoculation of the re-inoculation group (4.2.1.6.3.1) in Phase 1 of the experiment, another set of six, eight-week-old mice were housed in two cages of three mice each and inoculated with 0.1ml of a *M. haemomuris*-containing inoculum that had been generated in amplifier mice preceding Phase 1 of the experiment and prepared as inoculum aliquots (**Table 30**). Each of the inoculum aliquots used for primary inoculation of the re-inoculation group (**Table 33**) was also used to inoculate three re-inoculation control group mice immediately after completing the inoculation procedure in the re-

inoculation group. The re-inoculation control group was used to ensure inoculum viability throughout the inoculation procedure and monitor subsequent *M. haemomuris* infection and recovery from infection as frequent sampling of the re-inoculation group mice was avoided to minimise effects on cytokine expression. The origin (inoculum aliquot) and composition of the inocula used to inoculate the re-inoculation control group in Phase 1 is given in **Table 34**.

Mouse ID (re-inoculation control group)	Cage ID	Origin (inoculum aliquot)
Control 1 Control 2 Control 3	C2	Pool 1
Control 4 Control 5 Control 6	D2	Pool 2

Table 34 *Re-inoculation control group mice as inoculated to monitor primary M. haemomuris infection in Phase 1 of the cytokine expression study.*

4.2.1.6.3.3 Monitoring of M. haemomuris infection after Phase 1 inoculations

Following primary inoculation of the re-inoculation group (4.2.1.6.3.1), these mice were monitored for *M. haemomuris* infection at 0, 1, 2, 3, 7, 10 and 14 dpi by withdrawing 10µl of blood followed by *M. haemomuris*-specific qPCR as described above (3.2.2.3.2).

Following primary inoculation of the re-inoculation control group (4.2.1.6.3.2), these mice were monitored for *M. haemomuris* infection once daily at 0 to 14 dpi by withdrawing 10µl of blood followed by *M. haemomuris*-specific qPCR as described above (3.2.2.3.2).

Additionally, all inoculated mice were monitored for clinical signs at least once a day and three times daily around expected peak parasitaemia, approximately one to two weeks after inoculation (see infection kinetics described in Chapter 3; 3.3.2.2).

4.2.1.6.3.4 Naïve group

Forty-nine eight-week-old female mice were split into seven subgroups of seven mice each and housed in cages of four and three animals per subgroup. Mice '1' to '4' were housed in the first cage and mice '4' to '7' were housed in the second cage of each group (see cage allocations in **Table 33**). After an acclimatisation period of two weeks and coinciding with the primary *M. haemomuris* infection of the re-inoculation group (4.2.1.6.3.1), mice had 10µl of blood taken on days 0, 1, 2, 3, 7, 10 and 14 of the experiment, equivalent to dpi of the re-inoculation group, 4.2.1.6.3.1. Collected samples did not undergo further testing except for samples taken immediately prior to Phase 2 inoculations (presented later in 4.2.1.6.5). This sampling was performed to control for the possible effects of blood sampling (including wound healing, associated stress) on immunity in Phase 2 of the experiment.

4.2.1.6.3.5 Naïve control mice

Another set of six, eight-week-old mice were housed in cages of three mice each and subject to the same blood sampling regiment as the re-inoculation control group (once daily for 14 dpi, see 4.2.1.6.3.3). Collected samples did not undergo further testing except for samples taken immediately prior to Phase 2 inoculations (presented later in 4.2.1.6.4). The naive control group was used to monitor primary *M. haemomuris* infection in Phase 2 of the experiment (presented later in 4.2.1.6.5.2).

4.2.1.6.4 Recovery phase in-between Phase 1 and Phase 2

Fourteen days after Phase 1 inoculations (4.2.1.6.3.1 and 4.2.1.6.3.2), in the Recovery phase, the blood sampling frequency in the re-inoculation control group and naïve control group was reduced

and these mice were only sampled on days 21, 35, 42, 56, 98, 119 and 126 post Phase 1 inoculations. Samples from the re-inoculation control group were monitored for *M. haemomuris* infection using *M. haemomuris*-specific qPCR as described before (3.2.2.3.2) and samples from the naïve control group were not further analysed except samples taken 126 days post Phase 1 inoculations, to ensure *M. haemomuris*-negative status. The re-inoculation group and naïve group were not subject to any blood sampling during the Recovery phase.

4.2.1.6.5 Phase 2: Plasma cytokine expression in mice previously infected with *M. haemomuris* after re-inoculation with *M. haemomuris (re-inoculation group)* and naïve mice after primary *M. haemomuris* infection (naïve group)

4.2.1.6.5.1 Description of inocula used for re-inoculation of the re-inoculation group and primary infection of the naïve group

One-hundred and thirty-one days after primary inoculations in Phase 1 (4.2.1.6.3.1), six out of seven subgroups of the re-inoculation group were inoculated with 0.1ml of a *M. haemomuris*-containing inoculum that had been generated in amplifier mice preceding Phase 2 of the experiment and prepared in aliquots by pooling freshly collected, heparinised blood and diluting it with PBS as described in 4.2.1.6.2.4. The origin and composition of inoculation aliquots has been given in **Table 32** above. Re-inoculation of the re-inoculation group coincided with primary infection of the 49 naïve group mice (see **Figure 27**). At the day of re-inoculation of the re-inoculation group, six out of seven subgroups of the naïve group were inoculated with 0.1ml of the same *M. haemomuris*-containing inoculum aliquot that was used to inoculate the corresponding subgroups of the re-inoculation group. Three inoculation aliquots were used as inoculum during Phase 2 inoculations. The origin (inoculation aliquot) and composition of the inocula used to inoculate the re-inoculation group and naïve group in Phase 2 is given in **Table 35**.

Group inoculated	Mouse ID	Cage ID	*M. haemomuris* dose in 0.1ml of inoculum per mouse	Origin (inoculum aliquot)	Subgroup (exsanguination dpi)
			Phase 2		
Re-inoculation group	1, 2, 3, 4	A	-	-	0
	5, 6, 7	B			
	1, 2, 3, 4	C	1.39E+09	Pool 3	1
	5, 6, 7	D			
	1, 2, 3, 4	E	1.39E+09	Pool 3	2
	5, 6, 7	F			
	1, 2, 3, 4	G	1.06E+09	Pool 4	3
	5, 6, 7	H			
	1, 2, 3, 4	I	1.06E+09	Pool 4	7
	5, 6, 7	J			
	1, 2, 3, 4	K	1.08E+09	Pool 5	10
	5, 6, 7	L			
	1, 2, 3, 4	M	1.08E+09	Pool 5	14
	5, 6, 7	N			
			Phase 2		
Naïve group	1, 2, 3, 4	O	-	-	0
	5, 6, 7	P			
	1, 2, 3, 4	Q	1.39E+09	Pool 3	1
	5, 6, 7	R			
	1, 2, 3, 4	S	1.39E+09	Pool 3	2
	5, 6, 7	T			
	1, 2, 3, 4	U	1.06E+09	Pool 4	3
	5, 6, 7	V			
	1, 2, 3, 4	W	1.06E+09	Pool 4	7
	5, 6, 7	X			
	1, 2, 3, 4	Y	1.08E+09	Pool 5	10
	5, 6, 7	Z			
	1, 2, 3, 4	A2	1.08E+09	Pool 5	14
	5, 6, 7	B2			

Table 35 *Origin and composition of inocula used to inoculate the mice in Phase 2 of the cytokine expression study. On day 0 of Phase 2 (corresponding to 131 days after Phase 1 inoculations) the re-inoculation group mice and naïve group mice were inoculated with 0.1 ml of an inoculum that had been produced in aliquots (**Table 32**) by pooling and diluting blood from amplifier mice (**Table 31**). The experiment was split in two parts, Phase 1 and Phase 2 (inocula presented here) with an interspersed Recovery phase (see **Figure 27**). Phase 1 represents the primary M. haemomuris infection of the re-inoculation group and Phase 2 represents the re-inoculation of the re-inoculation group and coinciding primary infection of the naïve group. No mice were exsanguinated during Phase 1. In Phase 2, all mice from the re-inoculation group and naïve group were exsanguinated for collection of EDTA-plasma in groups of seven mice (equivalent to one subgroup) per day at 0, 1, 2, 3, 7, 10 and 14 dpi. Dpi, days post (Phase 2) inoculation.*

Mice were inoculated in sequential order of mouse '1' to '7' per subgroup and subgroups in the order of '1' to '14' using the technique described in 4.2.1.4.2. The order of inoculation of subgroups was matched across the main experimental groups; the identical subgroup was first inoculated in the re-inoculation group, then in the naïve group (i.e. subgroup '1' of the re-inoculation group was first inoculated, then subgroup '1' of the naïve group followed by subgroup '2' of the re-inoculation group, then subgroup '2' of the naïve group, etc.). Subgroups '0' did not receive any inocula during Phase 2. To ensure, inoculation aliquots did not change within the identical subgroups, the remaining inoculum was discarded if its volume dropped below 1.6ml. The higher volume cut-off compared to Phase 1 inoculations (0.8ml) was required to ensure subgroups of re-inoculation group mice and naïve group mice could be matched for inoculations as shown in **Table 35** above. Exsanguination of amplifier mice to generate an inoculation aliquot and inoculation of the same aliquot into experimental mice was completed within ten minutes.

4.2.1.6.5.2 Re-inoculation control group and naive control group: Description of inocula used for re-inoculation of the re-inoculation control group and primary infection of the naïve control group

After re-inoculation of the re-inoculation group and primary inoculation of the naïve group (4.2.1.6.5.1) in Phase 2 of the experiment, the six previously inoculated re-inoculation control group mice (4.2.1.6.3.2) and the six naïve control group mice (4.2.1.6.3.5) were inoculated with 0.1ml of a *M. haemomuris*-containing inoculum that had been generated in amplifier mice preceding Phase 2 of the experiment and prepared as inoculum aliquots (**Table 32**). Each of the inoculum aliquots used for Phase 2 inoculations (**Table 33**) was also used to inoculate one to three re-inoculation control group mice and one to three naïve control group mice immediately after completing the inoculation procedure in the main experimental groups. The varying number of inoculated mice was due to varying residual volumes of inoculation aliquots. The control groups were used to ensure inoculum viability throughout the inoculation procedure and monitor primary *M. haemomuris* infection in the naïve control group and protection from re-infection in the re-inoculation control group as sampling of the main experimental mice (re-inoculation group and naïve group) was avoided to minimise effects on cytokine expression. Origin (inoculation aliquot) and composition of inocula used to inoculate the re-inoculation control group and naïve control group are given in **Table 36**.

Mouse ID (naïve control group)	Cage ID	Mouse ID (re-inoculation control group)	Cage ID	Origin (inoculation aliquot)
Control 7	E2	Control 1	C2	Pool 3
Control 8	E2	Control 2	C2	Pool 4
Control 9		Control 3		
Control 10	F2	Control 4	D2	Pool 5
Control 11		Control 5		
Control 12		Control 6		

Table 36 *Re-inoculation control group mice and naïve control group mice as inoculated to monitor M. haemomuris infection in Phase 2 of the cytokine expression study. Control mice '1' to '6' had previously been inoculated with M. haemomuris as shown in* ***Table 34****.*

Inoculum aliquots were used in the order given by their use in the main experimental mice (4.2.1.6.5.1) whilst inoculating the re-inoculation control group mice prior to the naïve control group mice from the same inoculum aliquot to ensure optimal inoculum viability in the re-inoculation control group mice and avoid attributing protection from re-infection to reduced inoculum viability.

4.2.1.6.5.3 Monitoring of haemoplasma infection after Phase 2 inoculations

As blood sampling may have interfered with plasma cytokine expression, no daily sampling to monitor *M. haemomuris* infection was attempted from the re-inoculation group and naïve group mice (4.2.1.6.5.1 and **Figure 27**). Samples to determine *M. haemomuris* genome copies per µl blood by *M. haemomuris*-specific qPCR were obtained post-mortem from the main experimental mice, as will be described later in 4.2.2.2.2.

Mice from these two main experimental groups (re-inoculation group and naïve group) were subsequently killed in groups of seven mice per day on days 0, 1, 2, 3, 7, 10 and 14 after Phase 2 inoculations to collect EDTA-plasma for cytokine analysis as described later in 4.2.2.2.4 and 4.2.5.

Re-inoculation control group mice and naïve control group mice were monitored for *M. haemomuris* infection on days 1 to 14 post (Phase 2) inoculations (4.2.1.6.5.2) by *M. haemomuris*-specific qPCR. Additionally, they were monitored for clinical signs at least once a day and three times daily over expected peak parasitaemia approximately one to two weeks post inoculation (see infection kinetics

described in Chapter 3; 3.3.2.2). All mice from both control groups were exsanguinated under terminal anaesthesia at the end of the experiment, 14 days post Phase 2 inoculations.

4.2.2 Sample collection and processing of blood samples from laboratory mice

4.2.2.1 Sample collection during the haematology study

4.2.2.1.1 Collection of blood samples for qPCR from the amplifier mice

Samples from the amplifier mice preceding the haematology study (4.2.1.5.2) were collected once a day by venepuncture of the tail via a heparin-coated microcapillary tube and diluted in PBS as described previously (2.2.3.3.2.1). Samples were stored at -80°C until DNA extraction and qPCR.

4.2.2.1.2 Collection of blood samples for qPCR from the haematology study mice

Samples were collected by dipping an uncoated capillary tube (Hawskley & Sons) into the hub of the needle used for cardiac puncture (collected blood containing approximately 2.5mM EDTA) and allowing the tube to fill up to a pre-placed mark at 10µl. The capillary tube was then emptied into 90µl of PBS as described previously (2.2.3.3.2.1). Samples were stored at -80°C until DNA extraction and qPCR.

4.2.2.1.3 Collection of EDTA-anticoagulated whole blood for haematological analysis from the haematology group mice

Syringes (1ml; Becton Dickinson) were equipped with 5/8-inch 23G needles (Terumo) and preloaded with EDTA by drawing up a 25µl droplet of 50mM EDTA (Sigma-Aldrich) from a water-repellent surface. This procedure was completed on each day of EDTA-plasma collection, immediately prior to anaesthesia. Four mice were anaesthetised on days 0, 3, 7, 10 and 14 after inoculation of the haematology group mice and blood was collected by cardiac puncture as previously described (2.2.3.7), replacing heparinised syringes with the EDTA-preloaded syringes produced prior to anaesthesia. EDTA-anticoagulated whole blood samples were returned to the laboratory and stored at 4°C until analysis. Storage time was kept to a minimum to prevent reported changes in haematological parameters (361) and analysis was completed within a four-hour time window following sample collection on each study day.

4.2.2.2 Sample collection during the cytokine expression study

4.2.2.2.1 Collection of blood samples for qPCR from the amplifier mice

Samples from the amplifier mice preceding Phase 1 and Phase 2 of the experiment (4.2.1.6.2) were collected once a day by venepuncture of the tail via a heparin-coated microcapillary tube and diluted in PBS as described previously (2.2.3.3.2.1). Samples were stored at 4°C until DNA extraction and qPCR.

4.2.2.2.2 Collection of post-mortem blood samples for qPCR from the re-inoculation group and naïve group of the cytokine expression study on respective exsanguination days

Samples from the re-inoculation group and naïve group of the cytokine expression study were obtained during Phase 2 of the experiment (4.2.1.6.5) from seven mice per group on 0, 1, 2, 3, 7, 10 and 14 dpi. Blood was collected by dipping an uncoated capillary tube into the hub of the needle used for cardiac puncture (collected blood containing approximately 2.5mM EDTA) and allowing the tube to fill up to a pre-placed mark at 10µl. The capillary tube was then emptied into 90µl of PBS as described previously (2.2.3.3.2.1). Samples were frozen at -80°C until DNA extraction and qPCR.

4.2.2.2.3 Collection of blood samples for qPCR from the re-inoculation control group and the naïve control group (and sample collection during primary infection of the re-inoculation group in Phase 1)

Samples from the re-inoculation controls and naïve controls of the cytokine expression study were obtained once a day for 14 days following Phase 1 and Phase 2 inoculations. Additionally, samples were collected from the re-inoculation group during primary *M. haemomuris* infection on 0, 1, 2, 3, 7, 10 and 14 dpi. Blood samples were collected by venepuncture of the tail as described previously (2.2.3.6.1.1) but using uncoated capillary tubes that were allowed to draw up 1µl of 50mM EDTA (Sigma-Aldrich) immediately prior to the sampling procedure. Blood samples were then emptied into 90µl of PBS as previously described (2.2.3.3.2.1). Samples were then returned to the laboratory, transferred into 96-well plates and then frozen at -80°C until DNA extraction and qPCR.

4.2.2.2.4 Collection of EDTA-plasma from the re-inoculation group and naïve group of the cytokine expression study on respective exsanguination days

Syringes (1 ml; Becton Dickinson) were equipped with 5/8-inch 23G needles (Terumo) and preloaded with EDTA by drawing up a 25µl droplet of 50mM EDTA (Sigma-Aldrich) from a water-repellent surface. This procedure was completed on each day of EDTA-plasma collection, immediately prior to anaesthesia. Seven mice per group (re-inoculation group and naïve group) were anaesthetised on days 0, 1, 2, 3, 7, 10 and 14 post Phase 2 inoculations (4.2.1.6.5.1 and **Figure 27**) and blood was collected by cardiac puncture as described (31), replacing heparinised syringes with the EDTA-preloaded syringes produced prior to anaesthesia. EDTA-anticoagulated whole blood samples were returned to the laboratory and spun at 1000 x g for 10 min at 4°C. The plasma was removed carefully not to disturb the pellet. Samples were then returned to the laboratory and frozen at -80°C until cytokine analysis.

4.2.2.3 DNA Extraction of mouse blood samples for qPCR

4.2.2.3.1 DNA extraction from blood samples from amplifier mice

Diluted blood samples from all amplifier mice used in the haematology and cytokine expression studies (4.2.2.1.1 and 4.2.2.2.1) were always subject to qPCR analysis on the day of sample collection. DNA was extracted using single spin columns (Macherey-Nagel, UK) on each blood sampling day as previously described (2.2.1.2.1).

4.2.2.3.2 DNA extraction from blood samples from the re-inoculated group and naïve group of the cytokine expression study and blood samples from the haematology group of the haematology study

Diluted blood samples obtained post-mortem from the haematology group of the haematology study, the re-inoculation and naïve groups as well as samples obtained during the primary infection of the re-inoculation group of the cytokine study (4.2.2.1.2, 4.2.2.2.2 and 4.2.2.2.3) were kept frozen at -80°C until addition of lysis buffer was added during the DNA extraction process. Total nucleic acid (TNA) was extracted using an automated, magnetic bead system (Chemagic, Chemagen, UK). In brief, samples were diluted with PBS to a final volume of 200µl and incubated for 20 min at room temperature with protease (Chemagen) and lysis buffer (Chemagen). After incubation, the Chemagic instrument was used to add magnetic beads (Chemagen) to bind nucleic acids. Using the magnetic separator and two washing steps, the instrument eluted TNA in a final volume of 50µl elution buffer (Chemagen) and removed the magnetic beads. TNA samples were stored at -80°C until further use.

4.2.2.3.3 DNA extraction from blood samples from re-inoculation control group and naïve control group of the cytokine expression study

Diluted blood samples from the re-inoculation controls and naïve controls of the cytokine study (4.2.2.2.3) were processed as described for the mice comprising the main cytokine expression experiment (4.2.2.3.2), however half of the samples (0 to 7 dpi) were extracted on an earlier, separate occasion to allow for mid-experiment monitoring of successful *M. haemomuris* infection using the different inocula listed in 4.2.1.6.2.

4.2.3 Analysis of mouse blood samples for *M. haemomuris* genome copies

4.2.3.1 Conditions of the M. haemomuris-specific qPCR assay

Conditions of the *M. haemomuris*-specific duplex qPCR assay were identical to the conditions described in 3.2.2.3.2 using primer/probe combinations described in 3.3.1.4.3.

4.2.3.2 Analysis of qPCR data

Analysis of qPCR data, including normalisation and calculation of relative *M. haemomuris* genome copies per µl blood, was carried out as described for 3.2.2.3.2.1.

4.2.3.3 Statistical analysis of M. haemomuris genome copies in mouse blood samples

Mann-Whitney-U statistics were calculated to compare *M. haemomuris* genome copies per µl blood across groups, i.e. to compare *M. haemomuris* genome copies on individual days during primary *M. haemomuris* infection as needed to compare post-mortem qPCR results of the re-inoculation group or naïve group mice to their respective control groups.

Similarly, Mann-Whitney-U statistics were calculated on AUC values of individual mice to compare *M. haemomuris* genome copies per µl blood over the course of the first 14 dpi, i.e. to assess protective immunity as described in Chapter 3 (3.2.2.4.3) by using data from the re-inoculation control group and naïve control group. No AUC values were calculated for the haematology group of the haematology study, the re-inoculation group or the naïve group of the cytokine expression study as mice were only sampled once, at the time of exsanguination. SPSS v. 24 (IBM) was used for statistical analyses and significance was defined as $p<0.05$.

4.2.4 Haematological analysis

4.2.4.1 Sample preparation for haematology

EDTA-anticoagulated blood samples (4.2.2.1.3) were allowed to reach room temperature on a roller shaker at 40rpm prior to analysis.

4.2.4.2 Laboratory haematological analysis

Blood samples were subject to haematological analysis using the ADVIA 120 system (Siemens Healthcare GmbH, Erlangen, Germany) and its pre-configured software package (v. 1.2) with settings set to 'mouse'. Haematologic parameters were either measured directly or calculated. Directly measured analytes were red blood cell count (RBC), haemoglobin concentration (Hb), platelet count (PLT), total nucleated cell count (WBC) and absolute counts of reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells. Mean platelet volume (MPV), mean corpuscular volume (MCV), plateletcrit (PCT), haematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), cell haemoglobin concentration mean (CHCM), cell haemoglobin content (CH), haemoglobin concentration distribution width (HDW), red cell distribution width (RDW), platelet distribution width (PDW), lobularity index (LI), myeloperoxidase index (MPXI), white blood cell count by peroxidase (WBCP),

mean platelet component (MPC), mean platelet mass (MPM) and the percentages of reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells were calculated using the inbuilt software package.

4.2.4.3 Analysis of haematological data

As the haematology study (4.2.1.5) was a pilot study by design, descriptive statistics were used, and results were described as medians and ranges. Obtained values were compared to published normal values (95% confidence interval, CI) for adult, female C57BL/6 mice (362) and to the ranges of the samples obtained on day 0 of the experiment as an in-house, pre-infection control.

4.2.5 Cytokine analysis

4.2.5.1 Preparation of EDTA plasma samples

Samples were collected and processed as described in 4.2.2.2.4. Upon completion of the experiment, and after qPCR data from simultaneously collected blood samples was obtained to confirm *M. haemomuris* infection had been successful, EDTA-plasma samples were transported to the flow cytometry facility on dry ice, then allowed to defrost at 4°C and spun at 1000 x g for 10 minutes to pellet any remaining platelets and debris.

4.2.5.2 Selection of EDTA plasma samples for analysis with the cytokine immunoassay

One 96-well plate was available for cytokine analysis. Twelve wells were required for the assay standards and 84 wells were available for experimental plasma samples. Sample numbers per group were reduced to 6 mice per experimental day in the naïve group and 5 mice per experimental day in the re-inoculation group except for the day 0 subgroup that still comprised 6 samples. Sample selection was achieved semi-randomly, by excluding samples from mice that were known to have had increased haemolysis due to suboptimal sample collection (e.g. having to use a second syringe) and preferring samples of higher volume. A final list of included samples is given in **Table 37**.

Mouse ID	Cage ID	Mouse ID	Cage ID	Exsanguination group (dpi)
Re- inoculation group		Naïve group		
1, 2, 3 5, 6, 7	O P	1, 2, 3, 4 5, 6	A B	0
1, 2, 4 5, 6, 7	Q R	1, 2, 3, 4 7	C D	1
1, 2, 3 5, 6, 7	S T	1, 3, 4 5, 6	E F	2
1, 2, 4 5, 6, 7	U V	1, 2, 3 6, 7	G H	3
1, 2, 4 5, 6, 7	W X	1, 2, 3, 4 6	I J	7
1, 3, 4 5, 6, 7	Y Z	1, 2, 4 5, 6	K L	10
1, 2, 3, 4 5, 7	A2 B2	1, 3, 4 6, 7	M N	14

Table 37 *EDTA plasma samples included for cytokine analysis based on the experimental outline and inoculation schematic given in* ***Table 33****. Six mice per exsanguination group (defined by Phase 2 dpi) were included from the naïve group and the day 0 subgroup of the re-inoculation group. Five mice per exsanguination day were selected from remaining re-inoculation group mice. Dpi: days post (Phase 2) inoculation.*

4.2.5.3 Measuring cytokine concentrations using the Bio-Plex Pro™ 23-plex mouse cytokine immunoassay

4.2.5.3.1 Assay principle

The Bio-Plex Pro™ 23-plex cytokine immunoassay (Bio-Rad Laboratories Ltd., Watford, UK) detects and allows simultaneous detection and quantification of 23 mouse/rat cytokines from 12.5µl to 50µl of serum, plasma or culture supernatant. Magnetic beads, measuring 6.5µm are used to detect each cytokine. The beads are coupled with specific anti-cytokine antibodies and a specific fluorescent dye that allows assay (cytokine) identification. Beads are allowed to react with the sample and after removal of unbound protein (using the magnetic property of the beads to hold them in the wells) a biotinylated detection antibody is added to bind to a different epitope on the cytokine. Streptavidin-phycoerythrin (streptavidin-PE) is added to bind the biotin on the detection antibody and allow fluorescent identification. After washing, the reaction is measured in a flow-based bead suspension assay system and analytes are identified and quantified by bead colour and fluorescence intensity. All technical procedures were carried out in accordance with the manufacturer's instructions and are briefly outlined below.

4.2.5.3.2 Preparation of beads, standards and samples

In brief, antibody-coupled beads were carefully vortexed and diluted 1:10 to a total volume of 5.7ml. A four-fold dilution series of standards (6 dilutions and one blank) was prepared with standard diluent. Experimental EDTA-plasma samples were diluted 1:4 with standard diluent prior to analysis.

4.2.5.3.3 Preparation of detection antibody and streptavidin-PE

Detection antibodies were prepared 15 min prior to use by careful resuspension and diluting 1:10 with detection antibody diluent to a final volume of 3ml. Streptavidin-PE was prepared 10 minutes prior to use to a final volume of 6ml and vials were covered in aluminium foil to protect it from light.

4.2.5.3.4 Incubating magnetic beads, samples and standards

Magnetic beads were added to the plate and the plate washed twice with 100µl of wash buffer per well using a magnetic wash station to keep the beads in the wells. Experimental samples (equivalent to 12.5µl of EDTA-plasma) were added to the plate and standards were added in duplicate. No positive controls were included. This was followed by an incubation step of 30 minutes at room temperature on an orbital shaker at 850rpm.

4.2.5.3.5 Incubation of detection antibody

After bead incubation, the plate was washed three times with 100µl of wash buffer per well. Detection antibodies were added and allowed to incubate for 30 minutes at room temperature on an orbital shaker at 850rpm.

4.2.5.3.6 SA-PE incubation

After detection antibody incubation, the plate was washed three times with 100µl of wash buffer per well. Streptavidin-PE was added and allowed to incubate for 30 minutes at room temperature on an orbital shaker at 850rpm. This was followed by another wash step consisting of 3 x 100µl of wash buffer per well. Beads were subsequently resuspended with 125µl of assay buffer.

4.2.5.3.7 Detection of fluorescence

Fluorescence was detected using a Luminex® MAGPIX® platform (Luminex Corp, Austin, USA) measuring fluorescence, specific to the bead as excited by a laser at 621nm and the fluorescent streptavidin-PE (excited at 511nm) to report the amount of bound cytokine per bead.

4.2.5.4 Analysis of cytokine data

4.2.5.4.1 Evaluation of quantification (generation of optimised standard curve)

Multiplex assay performance was assessed using the Bio-Plex ManagerTM v. 6.1 software package (Bio-Rad). Recovery of standards (observed concentration/expected concentration x 100) was assessed for fitting within the expected recovery percentage. Outliers were identified as <10% CV (intraassay coefficient of variation, using standard duplicates) and excluded from generating an optimised standard curve for each cytokine. Five-parameter logistic regression (5PL) plots were generated using the five-parameter logistic equation to fit the standard data to a non-linear (sigmoid) regression model. Plots were assessed for location of sample values within the range of the standard curve and calculated values outside the standard curve marked for later analysis. Residual variance and probability of fit was assessed for each cytokine. Standard recovery was used as the primary measure as measurement quality as specified by the manufacturer.

4.2.5.4.2 Statistical analysis of cytokine multiplex data

Subsequent data analysis was performed using SPSS Statistics 24 (IBM). Analysis was performed in two stages, firstly, principal component analysis was performed to assess the intrinsic variation within the dataset across the 23 variables then reduce the dimensionality of the dataset based on extracted principal components. Secondly, univariate analyses of variance were conducted to assess the newly obtained variables (component scores) over time and between groups. Additionally, general linear models were built to better assess individual behaviours of cytokines, previously identified to be highly influential to the derived principal components.

4.2.5.4.2.1 Power calculations

No previous data were available to guide power calculations, so group sizes were based on maximum effect sizes as previously described (3.2.2.4.1), leaving the pilot nature of the study unchanged.

4.2.5.4.2.2 Pre-analytical considerations

Cytokine concentrations were recorded as pg/ml as converted from fluorescence values using the optimised standard curve (4.2.5.4.1). For cytokines whose values unexpectedly dropped below the limit of detection (i.e. when group medians were located well away from the limit of detection) the value was treated as an outlier and removed from analysis. However, to assess the potential introduction of bias, analysis was repeated, assuming '0pg/ml' for these outliers at the end of data analysis. Values located outside of the optimised standard curve were visually assessed and if found to be located within the well-defined linear part of the curve, included in analysis.

4.2.5.4.2.3 Calculation of percentage of change

Percentage of change over time was calculated for each absolute cytokine concentration, using the following formula:

$$\text{Percentage of change from baseline} = (C_{conc} - M^{d0})/M^{d0} \times 100$$

(C_{conc} = cytokine concentration of individual mouse, M^{d0} = Median of cytokine concentrations on day 0; the day 0 subgroup of the re-inoculation group was used for re-inoculation data and the day 0 subgroup of the naïve group was used for naïve group data respectively)

Subsequent analysis was performed on two datasets, comprising absolute values and percentage of change values respectively.

4.2.5.4.2.4 Principal component analysis (PCA)

A PCA was run for unsupervised clustering of data and hence unbiased identification of the main contributors to total variation in the multidimensional dataset of 23 cytokines in plasma and *M. haemomuris* genome copies in blood over time. This dataset was subsequently reduced to principal components which represent linear combinations of the original variables but aim to combine as much variation as possible within a single component, without necessarily reducing the information content. The first four extracted principal components were then further analysed in univariate analyses, aiming to define differences over time and across experimental groups. Univariate analysis hence was based on component scores, as they represent each individual cytokine result as it is plotted on the extracted component axis in the new dataset with reduced dimensions as described by DiStefano, Zhu (363). Cytokine concentrations and percentage of change from baseline (4.2.5.4.2.3) were analysed as two separate sets of variables and components were extracted from each set via PCA.

At this stage, the output of component loadings (describing the relationship between each measurement and the extracted component) is considered unrotated as it forces the reduced dimensions (principal components) to be orthogonal to each other. In other words, a dataset comprising three principal components would easily be visualised in a Cartesian coordinate system. Traditionally, rotation is performed on the component loading matrix to help interpretability. On a Cartesian coordinate system this means optimising the axes, so the components load in a more meaningful pattern. After rotation, dimensions may, but do not need to be orthogonal to each other which accounts for correlations between components. An unrotated component loading matrix was selected for initial interpretation and interpretability based on shared conceptual (immunological) meaning within and in-between components. Component loadings were then subject to Promax rotation to assess optimisation of interpretability by placing non-orthogonal axes to maximise component loadings on all extracted components. Extracted component scores, measuring each observation (i.e. cytokine concentration) on each of the four extracted components were saved for regression analysis.

Scree plots (which show the percentage of variance within the dataset that is explained by each extracted component) were generated to assess the number of meaningful components extracted. Components with an eigenvector >1 were extracted. The eigenvector reflects the direction of the extracted component with a dimensionless length that reflects the magnitude of the observed variation of the dataset it accounts for. Eigenvalues close to zero do not account for any significant variation within the dataset.

Appropriateness of PCA was assessed based on continuity and linear relationships between the measurements. Sampling adequacy was assessed using the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy as described by Kaiser (364). Sphericity of the data was tested using Bartlett's test of sphericity (365) which assesses adequate linear correlations between the variables and hence suitability of the dataset for reduction. Means and standard deviations were calculated for each of the extracted components and outliers were identified by component scores greater than three standard deviations from the mean if not already removed at pre-analytical stage (see 4.2.5.4.2.2). Scores for each component (akin to individual cytokine measurements in reduced dimensions) were plotted against each other to assess obvious clustering or divisions within the data.

4.2.5.4.2.5 Univariate analyses of variance

To further assess PCA results, general linear models were constructed, entering component scores as the dependent variable and experimental groups (re-inoculated and naïve) as well as dpi as fixed

factors. Significance was defined as p<0.05. Where significant effects between experimental groups were found, the analysis was repeated with the model allowing for linear interactions of experimental group and the dpi.

Additionally, general linear models were constructed for individual cytokines that were found to be loading on extracted components using the same approach as outlined for PCA factor scores.

4.2.5.4.2.5.1 Assessment of normality and residuals

For all univariate analyses, residuals (distance of observed datapoints to the value predicted by the linear model) were calculated and plotted against each other. Normality of errors and homogeneity of variance was assessed visually.

4.2.5.4.2.6 Comparison of individual days

Where appropriate, observed peaks in cytokine concentrations on individual days were assessed for significant differences against baseline concentrations using Mann-Whitney-U statistics. Significance was defined as $P_{MWU}<0.05$.

4.2.6 Post-mortem examinations

All mice killed in the experiments outlined above underwent a basic post-mortem examination. Splenomegaly was defined as >0.4% bodyweight (see 3.2.2.2.1). Spleens were removed and frozen separate to the carcasses at -80°C for potential future use.

4.2.6.1 Analysis of post-mortem data

Splenic weights at the time of post-mortem examination (0, 1, 2, 3, 7, 10 and 14 dpi) were calculated as % bodyweight and expressed as medians and ranges for each study group and dpi. Mann-Whitney-U statistics were calculated to compare splenic weights (as % bodyweight) between the naïve group and the re-inoculation group. Spearman's rank-order correlation coefficient (r_s) was calculated for observed splenic weight and *M. haemomuris* copies per µl blood using SPSS Statistics 24 (IBM SPSS Statistics for Windows, 24.0, IBM Corp., USA).

4.3 RESULTS

4.3.1 Clinical signs, deaths and post-mortem examinations

4.3.1.1 Haematology study

No clinical signs of ill health were noted in any of the mice throughout the experiment. Splenomegaly was documented in all 20 mice at post-mortem examination after being killed at 0, 3, 7, 10 and 14 dpi when using a 0.4% bodyweight cut-off to define splenomegaly. However, splenomegaly was only apparent in the second week post inoculation (mice killed at 7, 10 and 14 dpi) when splenic weights were compared to pre-inoculation weights, defined as the range of the mice killed at 0 dpi (without prior *M. haemomuris* inoculation) of the experiment (**Table 38**).

Subgroup (exsanguination dpi)	Number of mice examined	Splenic weight range (% bodyweight)	Splenic weight median (% bodyweight)
0	4	0.40 - 0.47	**0.43**
3	4	0.34 - 0.50	**0.43**
7	4	1.65 - 1.90	**1.85**
10	4	0.79 - 1.12	**0.88**
14	4	0.66 - 1.56	**0.84**

Table 38 *Splenic weights (as % bodyweight) in the mice killed at 0 (no inoculation), 3, 7, 10 and 14 days post inoculation with M. haemomuris in the haematology study. Four mice were killed at each study day. Values above normal (defined as the range of the mice killed at 0 dpi, without prior inoculation) are indicated by shaded cells. Bolded text indicates deviations based on a 0.4% bodyweight cut-off as defined by published confidence intervals for splenic size in female C57BL/6 mice (276). Dpi, days post inoculation.*

4.3.1.2 Cytokine expression study

No clinical signs of ill health were noted throughout the experiment. However, two mice, used as amplifiers preceding Phase 2 of the experiment (see 4.2.1.6.2.3 and **Figure 27**) were found dead at 8 and 10 dpi without any prior clinical signs being noted on monitoring that occurred three times per day. Post-mortem examinations of these mice revealed no abnormal finding other than splenomegaly with splenic weights of 1.98% bodyweight for the mouse found dead at 8 dpi and 1.65% for the mouse found dead at 10 dpi; the two mice had full stomachs, indicating the mice had not been inappetent prior to death.

During the main experiment (inoculation of re-inoculation group and naïve group and their respective control groups, see **Figure 27**) one mouse was found dead at 7 dpi (primary infection of the re-inoculation group, Phase 1, 4.2.1.6.3.1) before sample collection could be completed and a second mouse died peracutely at 8 dpi (primary infection of the naïve control group, Phase 2, 4.2.1.6.5.2). Post-mortem examinations of these mice revealed no abnormal findings other than splenomegaly with splenic weights comprising 1.95% bodyweight for the mouse found dead at 7 dpi and 2.04% bodyweight for the mouse found dead at 8 dpi) and some damage of the corpse by the remaining mice within the cage. These two mice were infected with *M. haemomuris* at different phases of the experiment and did not receive doses of the same inoculum. Splenic weights of the remaining mice of the cytokine expression study (including control groups killed 14 days after Phase 2 inoculations) are given in **Table 39**.

Subgroup (exsanguination dpi)	Number of mice examined	Splenic weight range (% bodyweight)	Splenic weight median (% bodyweight)	Subgroup (exsanguination dpi)	Number of mice examined	Splenic weight range (% bodyweight)	Splenic weight median (% bodyweight)
		Re-inoculation group				Naïve group	
0	7	0.41 – 0.65	**0.46**	0	7	0.35 – 0.46	**0.41**
1	7	0.45 – 0.60	**0.50**	1	7	0.34 – 0.49	**0.42**
2	7	0.40 – 0.57	**0.52**	2	7	0.35 – 0.47	0.40
3	7	0.42 – 0.59	**0.47**	3	7	0.42 – 0.52	**0.50**
7	6*	0.41 – 0.54	**0.44**	7	7	0.43 – 2.12	**1.65**
10	7	0.41 – 0.49	**0.45**	10	7	1.10 – 1.50	**1.33**
14	7	0.40 – 0.60	**0.47**	14	7	0.67 – 0.85	**0.77**
		Re-inoculation control group				Naïve control group	
14	6	0.41 – 0.57	**0.54**	14	5*	0.71 – 0.98	**0.78**

Table 39 *Splenic weights (as % bodyweight) in the mice killed in the cytokine expression experiment killed at 0 (no inoculation), 1, 2, 3, 7, 10 and 14 days post inoculation with M. haemomuris in Phase 2 of the cytokine expression study. Seven mice of each subgroup of the re-inoculation group and naïve group were killed on each study day except for subgroup '7' of the re-inoculation group where one mouse had died in Phase 1 of the experiment. Six mice were killed at 14 dpi from the re-inoculation control group and five mice (after one mouse had died at 8 dpi) were killed at 14 dpi from the naïve control group. Subgroups/groups with reduced mouse numbers due to death are indicated with an asterisk (*). The five naïve control group mice and six re-inoculation control group mice are presented separately as serial blood sampling may have influenced spleen size through extramedullary haematopoiesis. Values above normal (defined as the range of the mice killed at 0 dpi, without prior inoculation) are indicated by shaded cells. Bolded text indicates deviations based on a 0.4 % bodyweight cut-off as defined by published confidence intervals for splenic size in female C57BL/6 mice (276). Dpi, days post inoculation.*

Using Mann-Whitney-U statistics to compare splenic weights (as % bodyweight) between the naïve group and the re-inoculation group, splenic weights were not significantly different at 0 dpi (p_{MWU}=0.085). Following inoculation with *M. haemomuris,* using the same inoculum aliquot (see **Table 33**), splenic weight was significantly increased in the re-inoculation group at 1 dpi (p_{MWU}=0.004) and 2 dpi (p_{MWU}=0.006). There was no significant difference in splenic weights between groups at 3 dpi (p_{MWU}=0.565). Splenic weights were significantly increased in the naïve group at 7 dpi (p_{MWU}=0.010), 10 dpi (p_{MWU}=0.002) and 14 dpi (p_{MWU}=0.002).

4.3.2 Infection kinetics and protection from re-infection with *M. haemomuris*

4.3.2.1 Haematology study

4.3.2.1.1 Amplifier mice

None of the three amplifier mice preceding inoculation of the haematology group (see **Figure 26**) had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All three amplifier mice became successfully infected with *M. haemomuris* and showed infection kinetics similar to previous observations (see Chapter 3). Normalised relative *M. haemomuris* genome copies per µl blood in these amplifier mice, monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 28.**

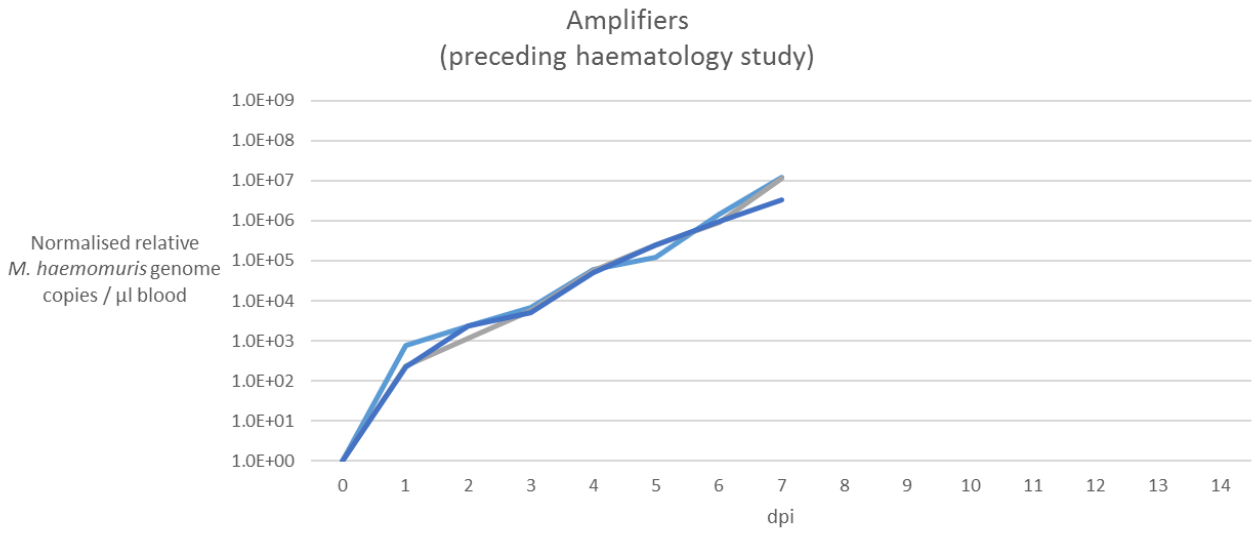


Figure 28 *M. haemomuris genome copies in the three amplifier mice preceding inoculation of the haematology group mice, following inoculation with fresh blood containing 6.91E+04 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copies per µl of blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily. One mouse (light blue line) was killed at 7 dpi and the two remaining mice not subject to daily blood sampling after 7 dpi. As zero values cannot be plotted on logarithmic scales, values of one (1.00E+00) haemoplasma copy per µl blood (and qPCR reaction) are treated as the equivalent of zero on this and on subsequent graphs within this chapter.*

4.3.2.1.2 Haematology group: Infection kinetics of primary *M. haemomuris* infection

None of the 20 haematology group mice had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All 20 mice successfully became infected with *M. haemomuris.* Normalised relative *M. haemomuris* genome copies per µl blood in the haematology group mice, monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 29**.

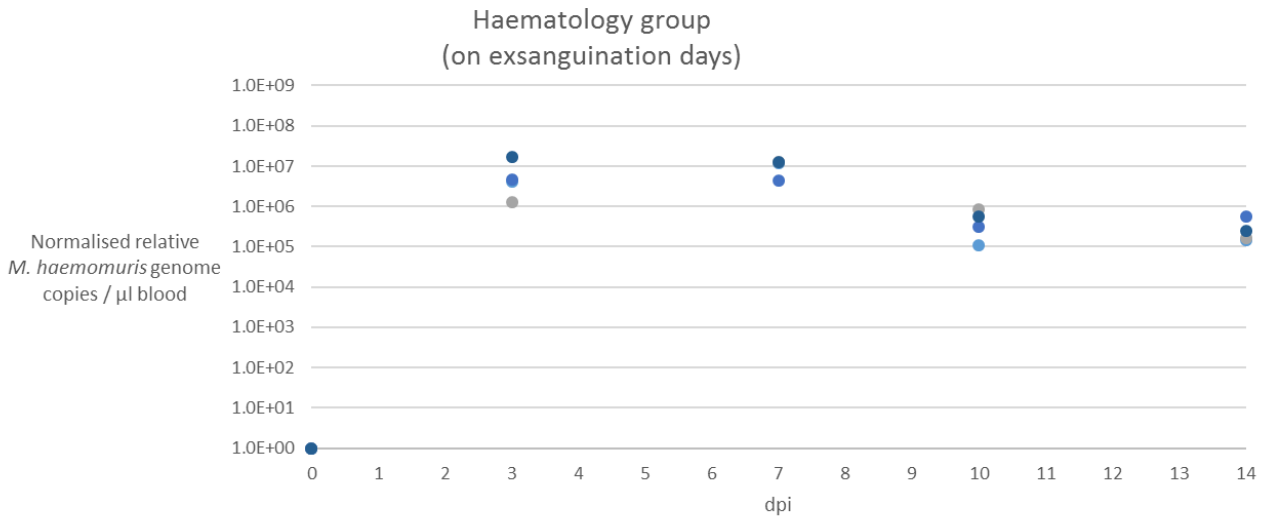


Figure 29 *M. haemomuris genome copies in the 20 haematology group mice undergoing primary M. haemomuris infection, following inoculation with freshly obtained blood containing 3.08E+08 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copies per µl of blood are plotted on the y-axis. Dots represent individual mice exsanguinated for haematological analysis on that day (one dot equals one mouse).*

The *M. haemomuris* genome copies per µl blood at the time of exsanguination agreed with previous observations of infection kinetics in mice naïve to *M. haemomuris* infection as described in Chapter 3 but no control group was monitored over the course of infection after inoculation from the same inoculum. However, observed *M. haemomuris* genome copies per µl blood in the 16 *M. haemomuris*-infected mice of the haematology group did not differ from age-matched, mice naïve to *M. haemomuris* infection that were inoculated as part of the cytokine expression experiment on respective sampling days of 0, 3, 7, 10 and 14 dpi (kinetics shown in **Figure 32** below). Mann Whitney U statistics comparing the *M. haemomuris* genome copies per µl blood on individual days for the haematology group and the naïve infection of the re-inoculation group of the cytokine expression study (4.2.1.6.3.1) showed no significant differences for day 0 (p_{MWU}=1.000), day 3 (p_{MWU}=0.055), day 7 (p_{MWU}=0.201), day 10 (p_{MWU}=0.806) and day 14 (p_{MWU}=0.327).

4.3.2.2 Cytokine expression study

4.3.2.2.1 Infection kinetics in amplifier mice preceding Phase 1

None of the eight amplifier mice preceding Phase 1 of the cytokine expression study (see **Figure 27**) had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All eight amplifier mice preceding Phase 1 of the cytokine expression study successfully became infected with *M. haemomuris* and showed infection kinetics similar to previous observations made in Chapter 3. Normalised relative *M. haemomuris* genome copies per µl blood in these eight amplifier mice, monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 30**.

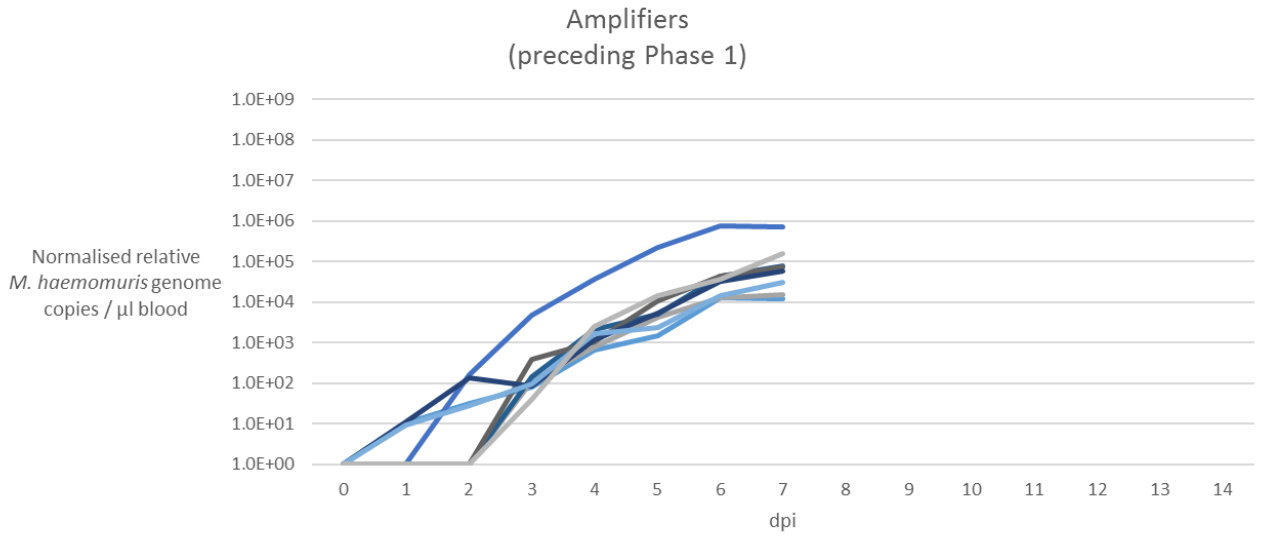


Figure 30 *M. haemomuris genome copies in the 8 amplifier mice preceding Phase1 of the cytokine expression study following inoculation with cryopreserved blood containing 2.63E+06 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copies per µl of blood are plotted on the y-axis. The individual mice are indicated by the colouring of the lines and represent individual mice sampled once daily. Six mice were killed 7 dpi and the two remaining mice not subject to daily blood sampling after 7 dpi.*

4.3.2.2.2 Infection kinetics in amplifier mice preceding Phase 2

None of the twelve amplifier mice preceding Phase 2 of the cytokine expression study (see **Figure 27**) had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All amplifier mice preceding Phase 2 successfully became infected with *M. haemomuris*. Normalised relative *M. haemomuris* genome copies per µl blood in these twelve amplifier mice, monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 31**.

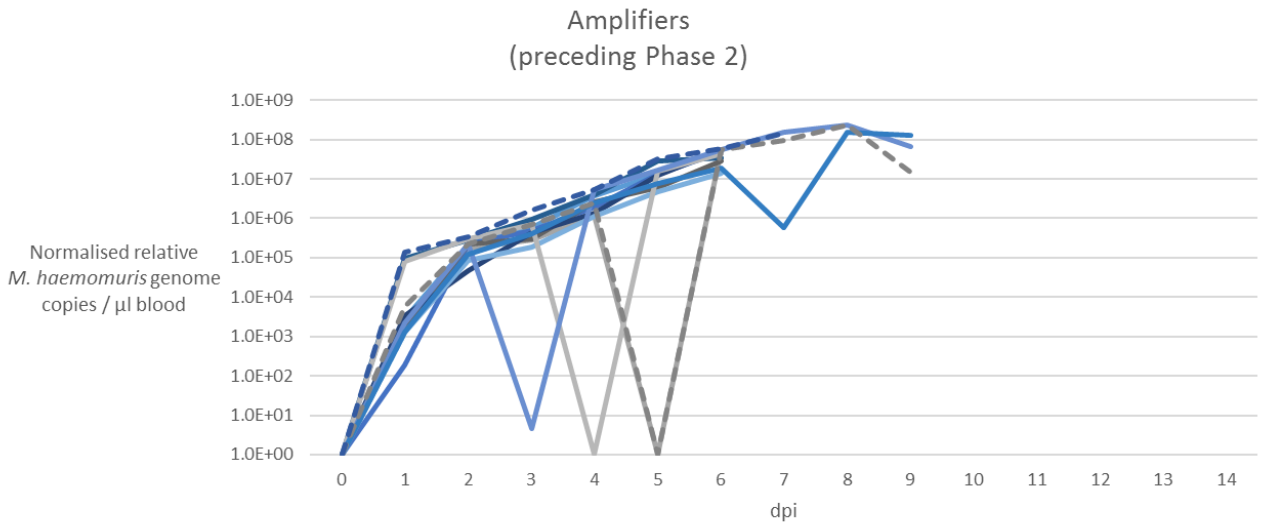


Figure 31 *M. haemomuris genome copies in the 12 amplifier mice preceding Phase 2 of the cytokine expression study following inoculation with freshly obtained blood containing 5.21E+06 M. haemomuris copies per inoculum dose. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copies per µl of blood are plotted on the y-axis. The individual mice are indicated by the colouring of the lines and represent individual mice sampled once daily. Dashed lines indicated the two mice found dead 8 and 10 dpi. Eight mice were killed 6 dpi (indicated by ending lines) and the remaining mice were killed on 10 dpi.*

Infection kinetics showed cycling of *M. haemomuris* genome copies in the bloodstream of four mice that had not previously been observed in *M. haemomuris* infection kinetics. The internal control (GAPDH) assay for the unexpectedly low or undetectable *M. haemomuris* genome copies fell within the expected range, ruling out extraction failure or qPCR inhibitors as a cause of the unusual cycling pattern (data not shown). The two amplifier mice found dead 8 and 10 dpi (dashed blue and grey lines in **Figure 31**) had 1.40E+08 and 1.43E+07 *M. haemomuris* copies per µl blood measured one day prior to death.

4.3.2.2.3 Infection kinetics of primary *M. haemomuris* infection (Phase 1: re-inoculation group and re-inoculation control group)

None of the 49 re-inoculation group mice and six re-inoculation control group mice had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All 49 re-inoculation group mice and six re-inoculation control group mice became infected with *M. haemomuris* and showed infection kinetics curves similar to previous observations in Chapter 3 (see 3.3.2.5). Normalised relative *M. haemomuris* genome copies per µl blood in the 49 re-inoculation group mice, monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 32, upper panel** and *M. haemomuris* genome copies per µl blood in the six re-inoculation control group mice, receiving doses of the same inoculum aliquots are shown in **Figure 32, lower panel.**

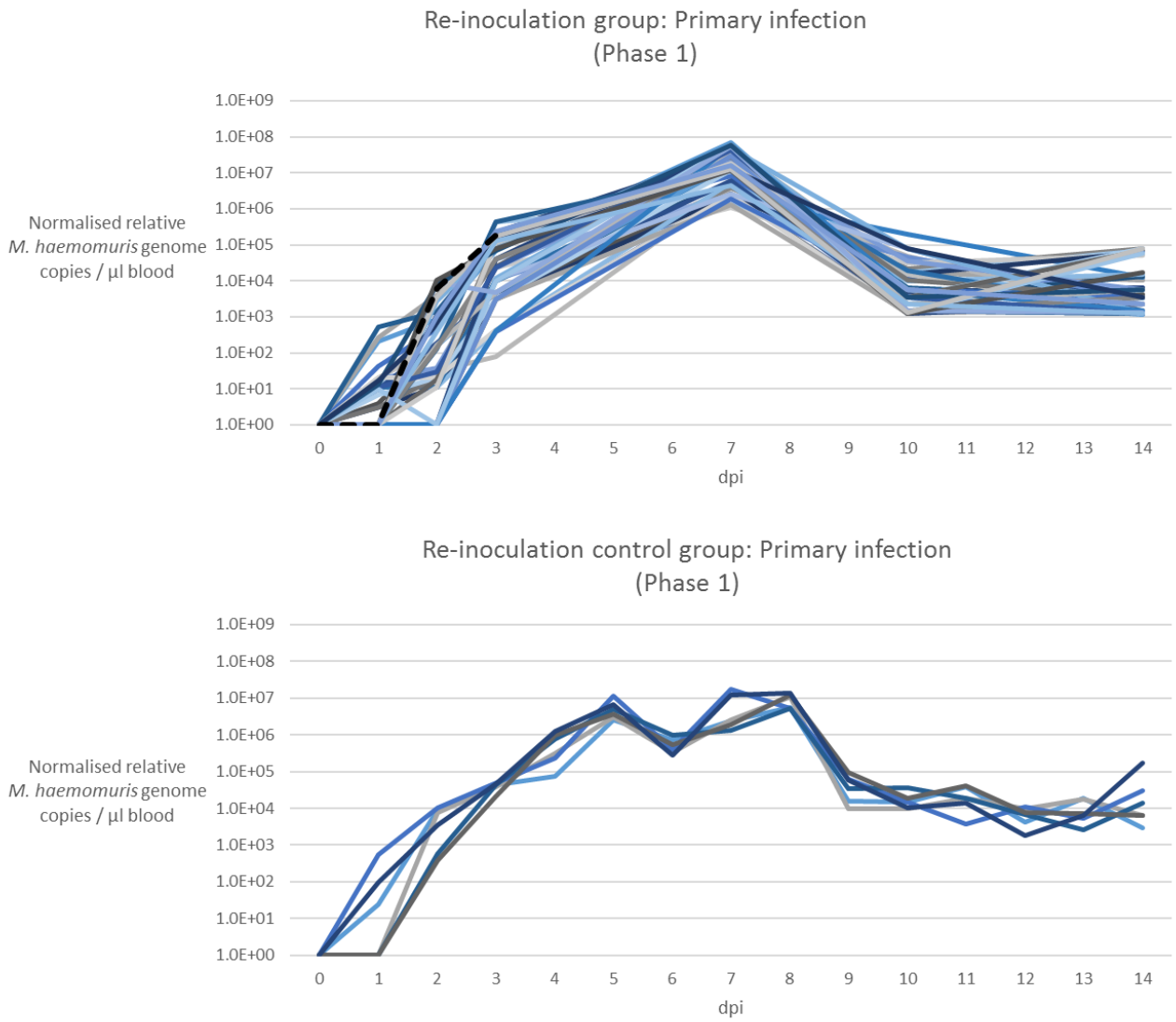


Figure 32 *M. haemomuris genome copies in the 49 re-inoculation group mice (upper panel) and the respective 6 re-inoculation control group mice (lower panel) showing the infection kinetics following primary inoculation with M. haemomuris in Phase 1 of the cytokine expression study after inoculation from the same inoculum aliquots as described in 4.2.1.6.3.1 and 4.2.1.6.3.2. The individual mice are indicated by the colouring of the lines and represent individual mice sampled at 0, 1, 2, 3, 7, 10 and 14 dpi (upper panel) and once daily (lower panel). The black dashed line (upper panel) indicates the mouse found dead at 7 dpi. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copy numbers per µl of blood are plotted on the y-axis.*

The mouse found dead at 7 dpi (indicated by the black dashed line in **Figure 32, upper panel** harboured 1.77E+05 *M. haemomuris* genome copies per µl blood on the last blood sample taken four days prior to death. As blood samples from the re-inoculation group were taken at 0, 1, 2, 3, 7, 10 and 14 dpi in Phase 1, no sample could be obtained ante mortem at 7 dpi.

The *M. haemomuris* genome copies per µl blood in the 49 re-inoculation group mice used for cytokine measurements (only sampled at 0, 1, 2, 3, 7, 10 and 14 post dpi in Phase 1) did not greatly differ from the genome copies measured in the six re-inoculation control group mice on the respective days of blood sampling. Mann-Whitney U statistics to compare *M. haemomuris* genome copies per µl blood between the re-inoculation group and the re-inoculation control group per sampling day showed no significant difference for day 0 (p_{MWU}=1.000, all negative), day 1 (p_{MWU}=0.345) , day 3 (p_{MWU}=0.409), day 7(p_{MWU}=0.065) day 10 (p_{MWU}=) and day 14 (p_{MWU}=0.085) post

inoculation but were significantly higher in the re-inoculation control group on day 2 (p_{MWU}=0.030) post inoculation.

Mycoplasma haemomuris genome copies per µl blood from the six re-inoculation control group mice during the Recovery phase in-between Phase 1 and Phase 2 of the experiment are shown in **Figure 33**.

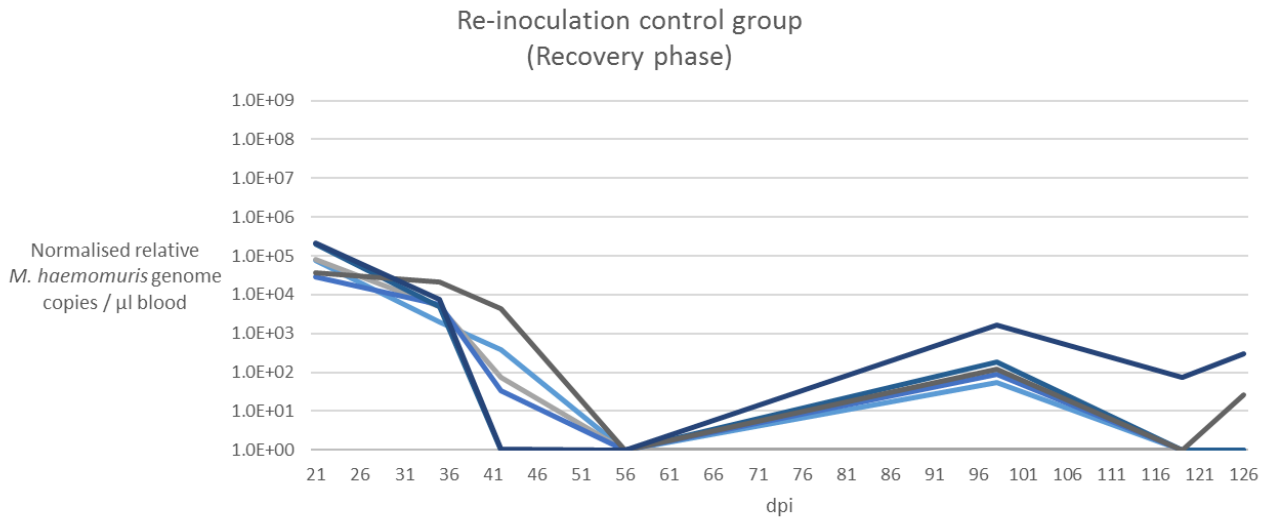


Figure 33 *M. haemomuris genome copies in the six re-inoculation control group mice during the Recovery phase following primary inoculation in Phase 1 of the cytokine expression study. The first 14 days of the infection kinetics curves have been shown in* ***Figure 32*** *above. The individual mice are indicated by the colouring of the lines and represent individual mice sampled at 21, 35, 42, 56, 98, 119 and 126 dpi. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copy numbers per µl of blood are plotted on the y-axis.*

During the Recovery phase, the re-inoculation control group showed low levels of *M. haemomuris* genome copies in their blood, but did not fully recover from infection, confirming previous observations made in Chapter 3.

4.3.2.2.4 Infection kinetics of primary *M. haemomuris* infection (Phase 2: naive group and naive control group)

None of the 49 naïve group mice and six naïve control group mice had haemoplasma DNA detected by the generic haemoplasma assay one week before entering the experiment. All mice successfully became infected with *M. haemomuris* and showed infection kinetics similar to previous observations in Chapter 3 (3.3.2.2). Normalised relative *M. haemomuris* genome copies per µl blood in the 49 naive group mice, monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 34**, **upper panel.** In Phase 2 of the cytokine expression study, blood samples from the naïve group were only collected at the time of exsanguination (4.2.1.6.5.3), meaning that individual dots (**Figure 34**, **upper panel)** represent individual mice and no lines were constructed. Normalised relative *M. haemomuris* genome copies per µl blood in the six naive control group mice, receiving doses of the same inoculum aliquots as the naïve group mice followed by daily blood sampling are shown in **Figure 34**, **lower panel.**

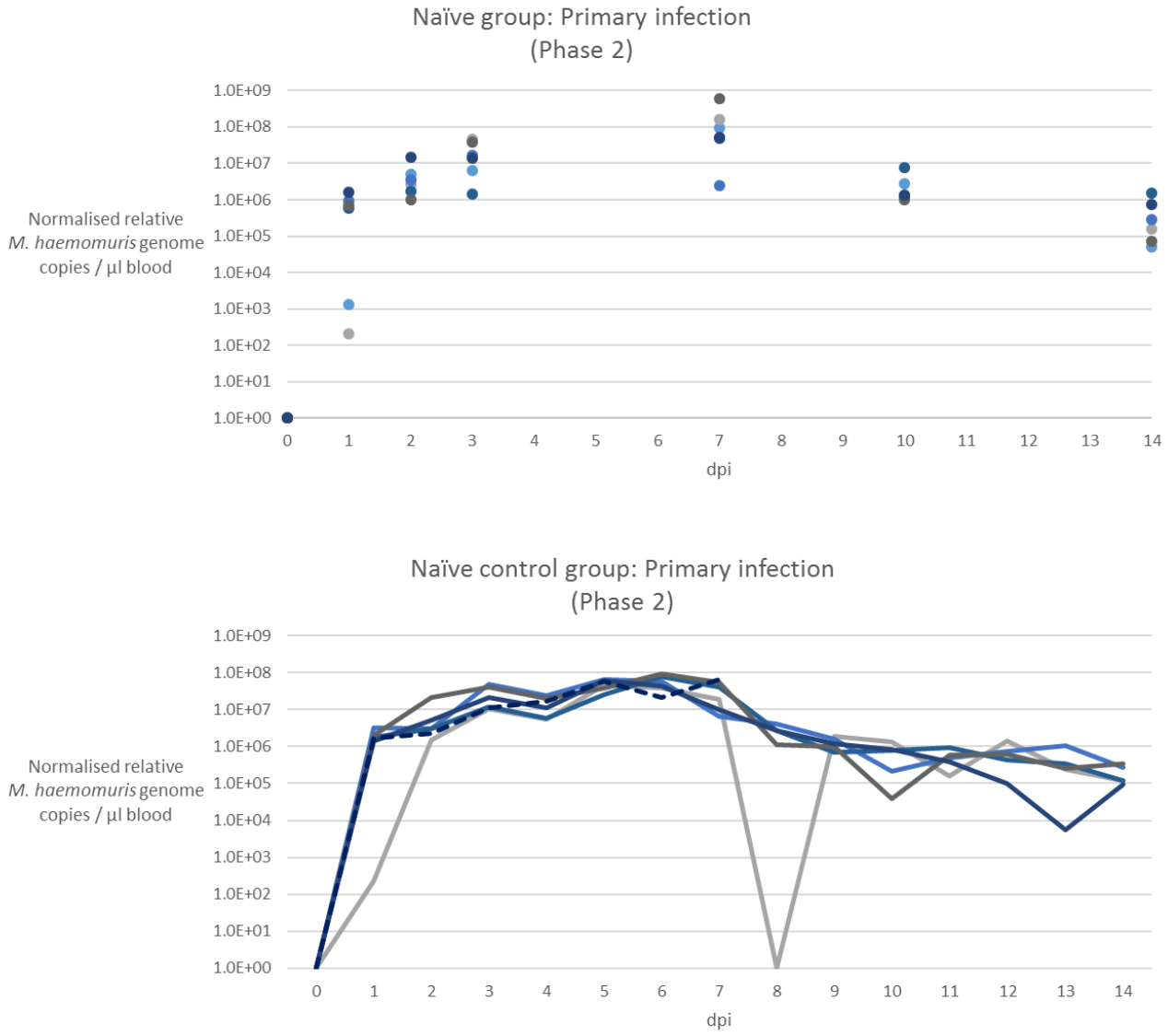


Figure 34 *M. haemomuris genome copies in the 49 naïve group mice (upper panel) and their respective six naïve control group mice (lower panel) showing the infection kinetics following primary inoculation with M. haemomuris in Phase 2 of the cytokine expression study after inoculation from the same inoculum aliquots as described in 4.2.1.6.5.1 and 4.2.1.6.5.2. Upper Panel: Individual dots represent individual mice at the time of exsanguination at 0, 1, 2, 3, 7, 10 and 14 dpi and no line charts were constructed. Lower panel: The individual mice are indicated by the colouring of the lines and represent individual mice sampled once daily. The dark blue dashed line (lower panel) indicates the mouse found dead at 8 dpi. Days post inoculation (dpi) are plotted on the x-axis and M. haemomuris genome copy numbers per µl of blood are plotted on the y-axis.*

The *M. haemomuris* genome copies per µl blood in the 49 naïve group mice used for cytokine measurements (only sampled on the day of exsanguination) did not differ greatly from the six naïve controls on respective blood sampling days. Mann-Whitney-U statistics to compare *M. haemomuris* genome copies between the naïve group and the naïve control group per sampling day showed no significant difference for day 0 (p_{MWU}=1.000, all negative), day 2 (p_{MWU}=0.749), day 3 (p_{MWU}=0.631), day 7 (p_{MWU}=0.200) and day 14: p_{MWU}=0.715) but was significantly lower in the naïve control group on day 1 (p_{MWU}=0.037) and higher in the naïve control group on day 10 (p_{MWU}=0.035).

4.3.2.2.5 Infection kinetics of partial protection from *M. haemomuris* re-infection (Phase 2: re-inoculation group and re-inoculation control group)

Normalised relative *M. haemomuris* genome copies per µl blood in the 48 (following one death in Phase 1) re-inoculation group mice, following re-inoculation with *M. haemomuris* in Phase 2 of the cytokine expression study and monitored using a *M. haemomuris*-specific qPCR assay are shown in **Figure 35**, **upper panel;** 0 dpi in Phase 2 is represented as 131 dpi in the graph for continuity. In Phase 2 of the cytokine expression study, blood samples from the re-inoculation group were only collected at the time of exsanguination (4.2.1.6.5.3), meaning that individual dots (**Figure 35**, **upper panel)** represent individual mice and no line charts could be constructed. Normalised relative *M. haemomuris* genome copies per µl blood in the six re-inoculation control group mice, receiving doses of the same inoculum aliquots as the re-inoculation group mice followed by daily blood sampling are shown in **Figure 35**, **lower panel.**

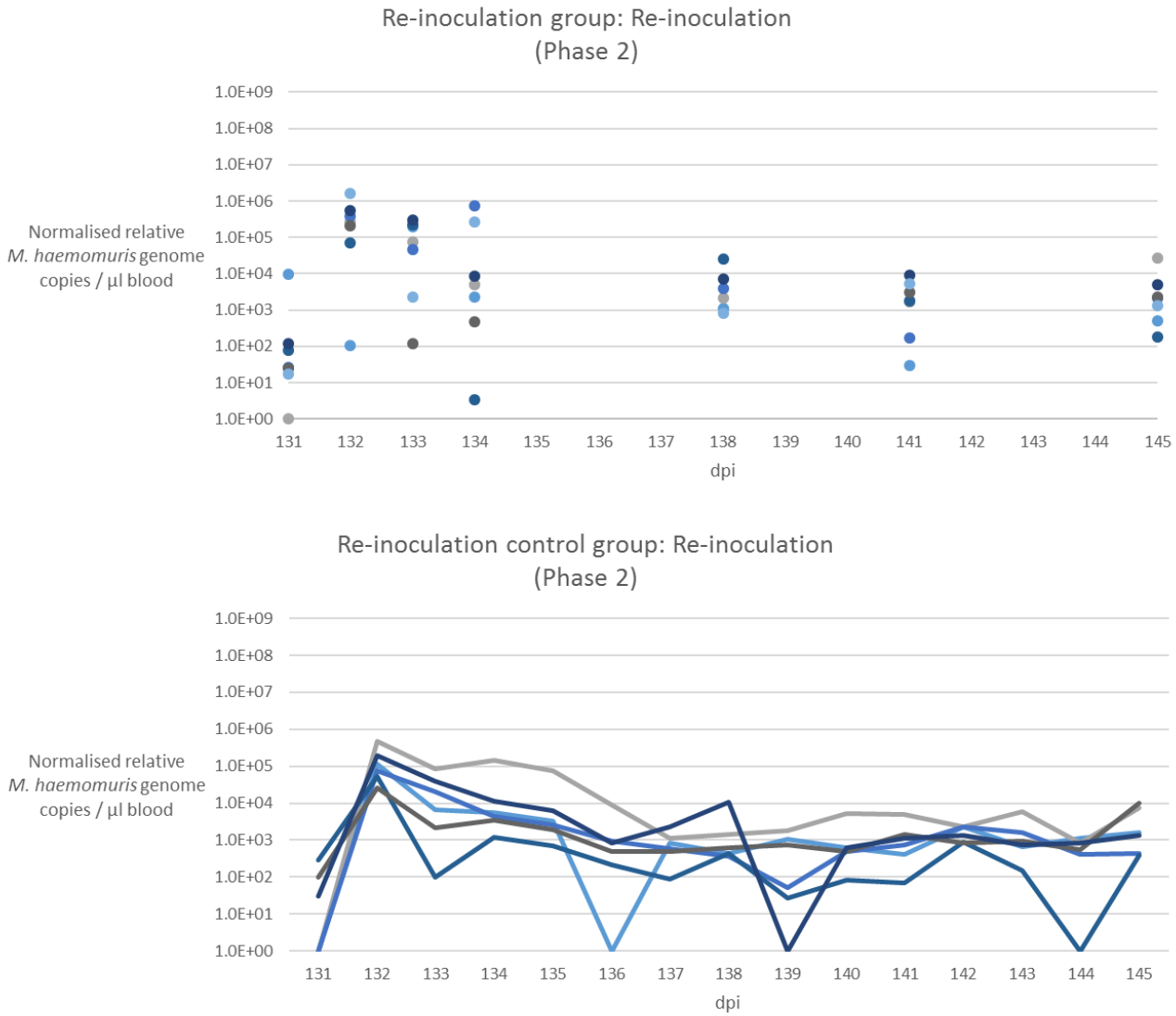


Figure 35 *M. haemomuris genome copies in the 48 re-inoculation group mice (upper panel) and their respective six re-inoculation control group mice (lower panel) showing the infection kinetics following re-inoculation with M. haemomuris in Phase 2 of the cytokine expression study after inoculation from the same inoculum aliquots as described in 4.2.1.6.5.1 and 4.2.1.6.5.2. Upper Panel: Individual dots represent individual mice at the time of exsanguination at 0, 1, 2, 3, 7, 10 and 14 dpi and no line charts could be constructed. Lower panel: The individual mice are indicated by the colouring of the lines and represent individual mice sampled once daily. Days post inoculation (dpi) are plotted on the x-axis. M. haemomuris genome copy numbers per µl of blood are plotted on the y-axis.*

The *M. haemomuris* genome copies in the 48 re-inoculation group mice used for cytokine measurements (only sampled at 0, 1, 2, 3, 7, 10 and 14 post dpi in Phase 2 where 0 dpi corresponds to 131 days post primary inoculation in Phase 1) did not greatly differ from the genome copies measured in the six naive control group mice on the respective days of blood sampling. Mann-Whitney-U statistics to compare *M. haemomuris* genome copies between the re-inoculation group and the re-inoculation control group per sampling day showed no significant difference for day 0 (p_{MWU}=0.514), day 1 (p_{MWU}=0.253) , day 2 (p_{MWU}=0.153), day 3 (p_{MWU}=0.886), day 10 (p_{MWU}=0.317) and day 14: p_{MWU}=0.775) but was significantly lower in the re-inoculation control group mice on day 7 (p_{MWU}=0.046).

The six re-inoculation control group mice (used to monitor infection kinetics by daily sampling whilst avoiding sampling of the re-inoculation group) demonstrated partial protection from re-infection, indicated as significantly lower *M. haemomuris* genome copies per µl blood (p_{MWU}=0.004, comparing AUC values over 14 dpi) than the six naïve control group mice (kinetics shown in **Figure 34**, **lower panel**) confirming previous results (3.3.2.5). No comparative statistics on protection from re-infection were performed on the main experimental mice, comparing the re-inoculation group and naïve group as these mice were used for cytokine measurements were only sampled on the day of exsanguination, delivering only single measurements, hence not allowing calculation of AUC values.

4.3.2.2.6 Correlation of splenomegaly and *M. haemomuris* genome copies

A Spearman's rank-order correlation was run between splenic weights at 14 dpi (as % bodyweight) and normalised relative *M. haemomuris* genome copies per µl blood at the time of exsanguination. There was a low but statistically significant positive correlation between splenic weight and *M. haemomuris* genome copies (r_s=0.412, p<0.001).

4.3.3 Haematological response to *M. haemomuris* infection

Problems with sample processing (clotting, n=1; machine repairs, n=2) led to three samples being lost for haematological analysis. This affected one sample, collected at 3 dpi and two samples collected at 14 dpi. Analysis of haematological parameters by comparing obtained values to published 95 % CI (362) yielded an unacceptable number of deviations in the 0 dpi group (which were considered a healthy, in-house control group) and a lack of sensitivity for changes over time. Preference was given to analysing the data by comparing group medians to ranges of the day 0 group to serve as a reference interval for each parameter within the experiment.

4.3.3.1 Haematocrit, haemoglobin and RBC

Red blood cell parameters over the course of naïve *M. haemomuris* infection as measured in four mice on 0, 7, 10 dpi, three mice on 3 dpi and two mice on 14 dpi are given in **Table 40**.

Parameter	unit	0 dpi (4 mice)	3 dpi (3 mice)	7 dpi (4 mice)	10 dpi (4 mice)	14 dpi (2 mice)
RBC	I.0E+12/L	8.595 (8.37-8.8)	8.75 (8.05-8.89)	**4.39 ↓ (4.06-4.75)**	7.72 ↓ (7.51-8.06)	8.39 (8.25-8.53)
HGB	g/dL	13.2 (12.8-13.3)	13.2 (12.2-13.3)	**6.85 ↓ (6.4-7.6)**	12.45 ↓ (11.8-12.9)	13.2 (13-13.4)
HCT	%	42.4 (39.9-43.4)	42.9 (38.9-43.3)	**22.4 ↓ (20.8-24.3)**	41.2 (38.9-42.5)	43.1 (42.6-43.6)
MCV	fL	49.2 (47.7-49.6)	48.7 (48.3-49.1)	51.25 ↑ (50.2-51.9)	52.8 ↑ (51.7-53.9)	51.35 ↑ (51.1-51.6)
MCH	pg	15.15 (15-15.7)	15.1 (14.9-15.2)	15.65 (15.6-16)	15.95 ↑ (15.7-16.3)	15.75 ↑ (15.7-15.8)
MCHC	g/dL	30.8 (30.4-33)	30.7 (30.6-31.5)	30.9 (30-31.2)	30.25 ↓ (29.8-30.7)	30.65 (30.6-30.7)
CHCM*	g/dL	30.05 (29.9-31.1)	30 (29.9-30.4)	31.2 ↑ (30.7-31.8)	30.55 (30.3-30.7)	30.35 (30.3-30.4)
CH*	pg	14.75 (14.6-14.9)	14.7 (14.5-14.7)	15.75 ↑ (15.7-15.8)	16.05 ↑ (15.6-16.5)	15.55 ↑ (15.5-15.6)
RDW	%	**12.45 (12.3-12.5)**	**12.3 (11.6-12.5)**	19.95 ↑ (18.9-23.3)	18.85 ↑ (18-19.1)	15.8 ↑ (15.5-16.1)
HDW*	g/dL	1.9 (1.88-1.98)	1.94 (1.93-1.97)	4 ↑ (3.86-4.11)	3.685 ↑ (3.46-3.99)	2.575 ↑ (2.57-2.58)
Morphology flags (noted/samples)	+/++/+++	-	Atypical + (1/3)	Anisocytosis +++ (4/4); Macrocytosis + (4/4); Hyperchromasia + (3/4); CH Variation +++ (4/4)	Anisocytosis +++ (4/4); HC Variation +++ (4/4)	Anisocytosis ++ (2/2)

Table 40 *Red blood cell parameters over the course of primary M. haemomuris infection measured from 2 to 4 mice exsanguinated 0, 3, 7, 10 and 14 days after primary inoculation with M. haemomuris. M. haemomuris infection kinetics curves have been presented in* ***Figure 29*** *above. Values are given as median (range) and derived from four mice per dpi, except 3 dpi (3 mice) and 14 dpi (2 mice). Values above or below normal (defined as the range of the mice killed at 0 dpi for each parameter) are indicated by ↑ or ↓ in shaded cells. Bolded text indicates deviations based on published confidence intervals for healthy, female C57BL/6. Asterisks (*) note parameters with no CI published by Charles River Laboratories (362). Abbreviations: RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; CHCM, cell haemoglobin concentration mean; CH, cellular haemoglobin content (mean of RBC cellular haemoglobin histogram); RDW, red cell distribution width; HDW, haemoglobin distribution width; Dpi, days post inoculation.*

There was a marked drop in RBC, HGB and HCT (all consistent with anaemia) at 7 dpi that coincided with peak parasitaemia (see **Figure 29** above). Erythrocyte indices were consistent with regeneration (i.e. decreased MCHC, increased MCH, MCV, RDW and HDW) following the onset of anaemia that were not fully resolved until the end of the experiment at 14 dpi. Erythrocyte morphology flags also agreed with a regenerative response indicated by variable levels of haemoglobin in variably sized cells. Haematological analysis based on laboratory CIs did reflect anaemia at 7 dpi but failed to indicate signs of regeneration as erythrocyte indices stayed within CIs despite a rather drastic increase in RDW – attributed to mice at 0 dpi all having values below published CIs, hence highlighting the increase over time.

4.3.3.2 Leucocyte parameters

Leucocyte parameters over the course of naïve *M. haemomuris* infection as measured in four mice at 0, 7, 10 dpi, three mice at 3 dpi and two mice at 14 dpi are given in **Table 41**.

Parameter	unit	0 dpi (4 mice)	3 dpi (3 mice)	7 dpi (4 mice)	10 dpi (4 mice)	14 dpi (2 mice)
WBC	I.0E+09/L	7.55 (6.47-8.37)	4.81 ↓ (4.47-5.19)	9.795 ↑ (9.12-13.09)	7.105 (5.86-9.86)	6.185 (5.94-6.43)
Neut	I.0E+09/L	**0.41 (0.34-0.49)**	**0.35 (0.31-0.45)**	0.84 ↑ (0.75-0.92)	0.7 ↑ (0.63-0.77)	**0.44 (0.37-0.51)**
Lymp	I.0E+09/L	6.045 (5.37-7.28)	3.81 ↓ (3.37-4.57)	8.425 ↑ (7.54-11.31)	5.97 (4.7-8.5)	5.315 ↓ (5.14-5.49)
Mono	I.0E+09/L	**0.155 (0.11-0.32)**	**0.08 ↓ (0.07-0.14)**	**0.12 (0.09-0.23)**	**0.115 (0.09-0.2)**	**0.16 (0.16-0.16)**
Eos	I.0E+09/L	0.265 (0.2-0.44)	0.22 (0.16-0.37)	0.17 ↓ (0.1-0.22)	0.12 ↓ (0.12-0.13)	0.205 (0.2-0.21)
Baso	I.0E+09 /L	0.035 (0.02-0.08)	0.03 (0.02-0.03)	0.05 (0.05-0.08)	0.03 (0.02-0.05)	0.02 (0.02-0.02)
LUC*	I.0E+09/L	0.265 (0.05-0.87)	0.15 (0.03-0.3)	0.33 (0.17-0.47)	0.21 (0.15-0.28)	0.05 (0.05-0.05)
LI*		2.04 (2-2.11)	1.98 ↓ (1.95-2)	2.04 (2.03-2.08)	1.955 ↓ (1.9-2.08)	2.015 ↓ (1.98-2.05)
MPXI*		1.75 (-0.7-11.2)	2.4 (-0.4-6.6)	-6.9 ↓ (-8.8- -1.5)	4.45 (0.3-7.5)	1.25 (1.2-1.3)
WBCP*	I.0E+09/L	7.095 (6.38-8.36)	4.85 ↓ (4.74-5.28)	9.995 ↑ (9.52-13.63)	7.195 (5.94-10.15)	6.245 ↓ (5.92-6.57)

Table 41 *Leukocyte parameters over the course of primary M. haemomuris infection measured from 2 to 4 mice exsanguinated 0, 3, 7, 10 and 14 days after primary inoculation with M. haemomuris. M. haemomuris infection kinetics curves have been presented in* ***Figure 29*** *above. Values are given as median (range) and derived from four mice per dpi, except 3 dpi (3 mice) and 14 dpi (2 mice). Values above or below normal (defined as the range of the mice killed at 0 dpi for each parameter) are indicated by ↑ or ↓ in shaded cells. Bolded text indicates deviations based on published confidence intervals for healthy, female C57BL/6. Asterisks (*) note parameters with no CI as per Charlesriver laboratories. Abbreviations: WBC, white blood cells; Neut, neutrophils; Lymp, lymphocytes; Mono, monocytes; Eos, eosinophils; Baso, basophils; LUC, large unstained cells; LI, lobularity index; MPXI, myeloperoxidase index; WBCP, white blood cell count by peroxidase; Dpi, days post inoculation.*

There was an increase in white blood cells (lymphocytes and neutrophils) suggesting an inflammatory response to infection and antigenic stimulation of lymphocytes. The preceding lymphopenia at 3 dpi is consistent with corticosteroid release, either mediated by the start of the experiment or acute onset of infection as increasing *M. haemomuris* genome copies per µl blood at the time suggest (

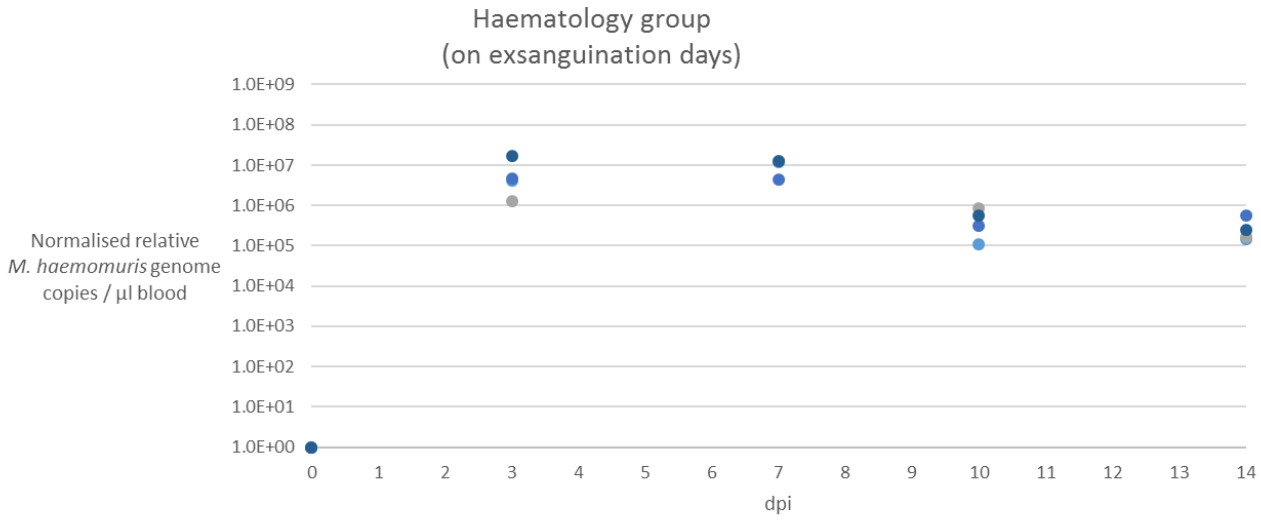


Figure 29). A similar reaction pattern was observed for eosinophils in a later stage of the infection. A drop in neutrophil MPXI is consistent with bacterial sepsis at day 7 post inoculation. Peroxidase-derived white blood cell counts (WBCP) were similar to WBC, indicating no major morphologic abnormalities but due to low sample numbers no statistical comparisons were attempted.

Haematological analysis based on laboratory CIs did not reflect changes in leucocyte parameters as mice at 0 dpi had below-normal neutrophil counts that only rose to remain within CI. All mice were classed as monocytopenic based on laboratory CIs.

4.3.3.3 Platelet parameters

Platelet counts and indices over the course of naïve *M. haemomuris* infection as measured in four mice on 0, 7, 10 dpi, three mice on 3 dpi and two mice on 14 dpi are given in **Table 42**.

Parameter	Unit	0 dpi (4 mice)	3 dpi (3 mice)	7 dpi (4 mice)	10 dpi (4 mice)	14 dpi (2 mice)
PLT	I.0E+09/L	1077.5 (523-1226)	950 (342-1050)	623.5 (614-641)	699.5 (635-713)	1102 (1057-1147)
MPV	fL	**7.6 (6.6-7.8)**	**7.2 (7-7.5)**	**7.25 (7-7.9)**	**7.35 (7.1-7.9)**	**8.35 ↑ (8.1-8.6)**
PDW*	%	46.45 (40.2-55.6)	45.8 (44.7-46.1)	41.4 (40.4-44)	45.55 (43.4-46.1)	49.45 (48.6-50.3)
PCT*	%	0.755 (0.41-0.96)	0.69 (0.24-0.79)	0.455 (0.43-0.51)	0.51 (0.49-0.53)	0.92 (0.86-0.98)
MPC*	g/dL	21 (20.6-23.4)	21.5 (21.5-21.9)	23 (21.7-23.8)	22.7 (21.2-23.8)	20.9 (20.3-21.5)
MPM*	Pg	1.415 (1.4-1.43)	1.42 (1.37-1.44)	1.565 ↑ (1.55-1.58)	1.52 ↑ (1.51-1.53)	1.515 ↑ (1.51-1.52)
Large Plt*	I.0E+09/L	5.5 (1-8)	3 (1-3)	2 (1-4)	2.5 (2-4)	16 ↑ (12-20)

Table 42 *Platelet parameters over the course of primary M. haemomuris infection measured from 2 to 4 mice exsanguinated 0, 3, 7, 10 and 14 days after primary inoculation with M. haemomuris. M. haemomuris infection kinetics curves have been presented in* ***Figure 29*** *above. Values are given as median (range) and derived from four mice per dpi, except 3 dpi (3 mice) and 14 dpi (2 mice). Values above or below normal (defined as the range of the mice killed at 0 dpi for each parameter) are indicated by ↑ or ↓ in shaded cells. Bolded text indicates deviations based on published confidence intervals for healthy, female C57BL/6. Asterisks (*) note parameters with no CI as per Charlesriver laboratories. Abbreviations: PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; MPC, mean platelet component (measure of activation); MPM, mean platelet mass; Dpi, days post inoculation.*

Platelet counts stayed within the range observed at 0 dpi and published normal values throughout the experiment. There was an elevation in platelet volume (MPV) and mass (MPM) following 7 dpi, with a marked increase in large platelets at the last day of the experiment (14 dpi).

Haematological analysis based on laboratory CIs consistently reported elevated MPVs but no changes over time.

4.3.4 Plasma cytokine expression in response to primary *M. haemomuris* infection and re-inoculation with *M. haemomuris* (occurring with partial protection from re-infection)

Forty-two EDTA plasma samples were analysed from the naïve group (six samples from each exsanguination day; 0, 1, 2, 3, 7, 10 and 14 dpi) during the first two weeks following primary *M. haemomuris* inoculation and 36 EDTA plasma samples were analysed from the re-inoculation group (six samples collected at 0 dpi and five samples collected 1, 2, 3, 7, 10 and 14 dpi) during the first two weeks following re-inoculation with *M. haemomuris*. All plasma samples were collected during Phase 2 of the cytokine expression experiment (see **Figure 27**).

4.3.4.1 Evaluation of quantification

Quantification of cytokines was evaluated according to the manufacturer's instructions (Bio-Rad Laboratories). Recovery of standards fell within the acceptable 70-130% range for all but the most dilute standard for GM-CSF which was still included for analysis. Thirteen standard values were classified as outliers and excluded from the standard curve generation for each cytokine: Standard 1 (highest concentration) was excluded for GM-CSF, IL-9, MCP-1, MIP-1a and RANTES. Standard 2 was excluded for GM-CSF. Standard 8 (lowest concentration) was excluded for IL-12(p70), IL-13, KC, Eotaxin, IL-4, IL-1a and IL1-b.

4.3.4.2 Principal Component Analysis

4.3.4.2.1 Suitability of data

The dataset was found suitable for PCA. The KMO measure of sampling adequacy was 0.825 (values closer to 1 mean better fit) and Bartlett's test demonstrated sphericity of the data with $p<0.001$.

4.3.4.2.2 Extraction of principal components and component scores

The first four components displayed eigenvalues of >1 which was consistent with the interpretation of the Scree plot (Appendix C, **Figure C-1**), suggesting only the first four components were meaningful to the analysis. Components 1 to 4 accounted for 77.9% of the observed variance within the dataset as shown in **Table 43**.

Component	Eigenvalue	% of Variance explained	Cumulative % of Variance explained
1	11.042	48.009	48.009
2	3.694	16.062	64.070
3	1.866	8.112	72.182
4	1.315	5.718	**77.900**

Table 43 *Total variance of the dataset of 23 cytokines from 42 mice undergoing naïve M. haemomuris infection and 36 mice undergoing re-infection with M. haemomuris as variance is explained by principal component 1 - 4, using the eigenvalue > 1 criterion of selection. Eigenvalues represent the (dimensionless) length of the eigenvector. Eigenvectors are used to define the direction of the variation within the data after dimension reduction. Similar eigenvalues do not need to represent similar variance as they represent a different dimension, orthogonal to the other components. Eigenvalues close to zero do not account for any significant variation within the data respectively.*

Four principal components were extracted and respective component loadings (representing the relationship of the underlying variable i.e. cytokine concentration to the extracted component) are shown as a component matrix in **Table 44**.

Cytokine	Component loadings			
	Component 1	Component 2	Component 3	Component 4
IL-1a	**0.558**	0.428	0.363	0.157
IL-1b	**0.803**	-0.482	0.159	0.002
IL-2	0.216	**0.774**	0.375	-0.109
IL-3	**0.905**	-0.165	-0.048	0.299
IL-4	**0.900**	0.094	0.126	0.110
IL-5	**0.815**	-0.337	-0.039	0.026
IL-6	**0.738**	-0.107	-0.110	0.443
IL-9	**0.919**	-0.142	0.214	-0.037
IL-10	**0.563**	0.310	-0.382	-0.504
IL-12p40	0.486	**0.553**	-0.390	-0.156
IL-12p70	**0.810**	-0.031	0.205	-0.139
IL-13	0.493	-0.294	0.454	-0.161
IL-17	-0.074	**0.882**	0.145	0.094
Eotaxin	-0.487	0.370	0.338	0.310
GCSF	**0.552**	0.294	-0.406	0.223
GMCSF	**0.875**	-0.222	-0.095	0.094
IFN-γ	**0.912**	0.080	0.238	0.002
KC	0.465	-0.096	**0.670**	-0.364
MCP1	**0.694**	0.146	-0.059	-0.295
MIP1a	**0.788**	0.220	-0.300	-0.342
MIP1b	**0.820**	0.309	-0.297	0.102
RANTES	-0.075	**0.881**	0.163	0.103
TNF-α	**0.873**	0.050	-0.039	0.367

Table 44 *Component matrix of component loadings from individual cytokine variables as extracted by principal component analysis (PCA) for components with eigenvalues > 1. Component loadings represent the relationship between the original variable and the extracted component. (Squared component loadings give the percentage of variance that is explained by the original variable prior to dimension reduction.) Component loadings were considered significant for their respective components if they exhibited a loading greater than 0.5 for that component and less than 0.5 for all other components. These are indicated by shaded cells and bolded text. Component 4 was included in PCA interpretation to guide further analysis of individual cytokine responses. IL-1a, Interleukin-1a; IL-1b, Interleukin-1b; IL-2, Interleukin-2; IL-3, Interleukin-3; IL-4, Interleukin-4; IL-5, Interleukin-5; IL-6, Interleukin-6; IL-9, Interleukin-9; IL-10, Interleukin-10; IL-12p40, Interleukin-12 Subunit p40; IL-12p70, Interleukin-12 Subunit p70; IL-13, Interleukin-13; IL-17, Interleukin-17; GCSF, Granulocyte Colony Stimulating Factor; GMCSF, Granulocyte-Macrophage Colony Stimulating Factor; IFN-γ, Interferon Gamma; KC, Keratinocyte Chemoattractant (CXCL1); MCP1, Mast Cell Protease 1 (CCL2); MIP1a, Macrophage Inflammatory Protein 1a; MIP1b, Macrophage Inflammatory Protein 1b; RANTES, Chemokine 'Regulated on Activation, Normal T cell Expressed and Secreted', (CCL5); TNF-α, Tumour Necrosis Factor Alpha.*

In interpreting the (unrotated) component matrix (**Table 44**), a cytokine was interpreted to load on a given component if the component loading was greater than an arbitrarily chosen value of 0.5 for that component and less than 0.5 for the remaining components. Employing these criteria, 16 cytokines were found to load on component 1, four cytokines were found to load on component 2, one cytokine loaded on component 3 and none on component 4. Component loadings, derived from percentage of change from baseline instead of absolute values of cytokine concentration did not exhibit a pattern different to the presented loadings from absolute values and were not further explored in the analysis but are given in Appendix C, **Table A-C1**.

4.3.4.2.3 Univariate analyses of unrotated component scores

Unrotated component scores, representing each measurement on the extracted component from the unrotated solution were calculated for each individual cytokine measurement for further investigation of the dataset. The distribution of errors and homogeneity of variance was consistent with normal distribution and homogeneity as assessed visually (data not shown). Results of univariate analyses on the first four unrotated component scores (assigning each individual cytokine value a score on a given component) and experimental groups (re-inoculation group versus naïve group), time (dpi) and interactions between the experimental group and dpi are presented in **Table 45** and visualised in **Figure 36**.

Dependent variable:		Component scores on principal component 1			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	26.824	13	2.063	2.978	**0.003**
Intercept	0.542	1	0.542	0.782	0.381
Group	9.855	1	9.855	14.225	**0.000**
Dpi	9.604	6	1.601	2.310	**0.050**
Group * Dpi	7.017	6	1.169	1.688	0.146
Error	31.176	45	0.693		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .462 (Adjusted R Squared = .307)					
Dependent variable:		Component scores on principal component 2			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	21.205	13	1.631	1.995	**0.044**
Intercept	0.011	1	0.011	0.014	0.908
Group	11.983	1	11.983	14.655	**0.000**
Dpi	4.621	6	0.770	0.942	0.475
Group * Dpi	3.607	6	0.601	0.735	0.624
Error	36.795	45	0.818		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .366 (Adjusted R Squared = .182)					
Dependent variable:		Component scores on principal component 3			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	33.088	13	2.545	4.597	**0.000**
Intercept	0.394	1	0.394	0.711	0.403
Group	1.289	1	1.289	2.328	**0.134**
Dpi	17.522	6	2.920	5.275	**0.000**
Group * Dpi	11.703	6	1.951	3.523	**0.006**
Error	24.912	45	0.554		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .570 (Adjusted R Squared = .446)					
Dependent variable:		Component scores on principal component 4			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	24.572	13	1.890	2.544	**0.010**
Intercept	0.006	1	0.006	0.008	0.929
Group	1.346	1	1.346	1.812	**0.185**
Dpi	2.567	6	0.428	0.576	**0.747**
Group * Dpi	22.147	6	3.691	4.969	**0.001**
Error	33.428	45	0.743		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .424 (Adjusted R Squared = .257)					

Table 45 *Univariate analyses of component scores (dependent variable) and experimental groups (naïve group versus re-inoculation group) and dpi (dpi in Phase 2 of the cytokine expression study, see* ***Figure 27****). Significance was defined as p<0.05 and significant results are indicated by shaded cells and bolded text, results that became significant due to the interaction effect are indicated by bolded text alone. Component scores 1 and component scores 2 were significantly different between the groups and bordered significance levels for dpi on component scores 2. Significant linear interactions (Group * Dpi) were found for component scores 3 and component scores 4. A visualisation of the component scores on all four principal components the 14-day observation period is given in* ***Figure 36****. Note, that component scores reflect cytokine concentrations but cannot be interpreted as such. Group, experimental group (naïve group versus re-inoculated group); df, degrees of freedom; F, F-statistic; Dpi, days post inoculation.*

Component scores on principal component 1 (see interpolation lines in **Figure 36, top left panel** too) were found to be significantly higher in the re-inoculation group ($p<0.001$) and approaching significance levels ($p=0.050$) for a difference over time, defined as days post inoculation (dpi). However, the chronological order of changes in cytokine concentrations was not assessed by the current statistical analysis. No linear interaction effect was found ($p=0.146$), meaning that the difference over time was not significantly difference between groups. Component scores on principal component 2 (see interpolation lines in **Figure 36, top right panel**) were significantly higher in the naïve group ($p<0.001$) with no linear interaction effect with dpi ($p=0.624$), meaning that the difference stayed the same over the observation period when both groups were assessed. Component scores on principal component 3 (see interpolation lines in **Figure 36, middle left panel**) were significantly different over time ($p<0.001$) and dependent on the experimental group with $p=0.006$ for the interaction effect of group and dpi, meaning that component scores changed in different directions in the re-inoculated group at different time points. Component scores on principal component 4 (see interpolation lines in **Figure 36, middle right panel**) displayed a significant interaction effect ($p=0.001$).

Adding normalised, relative *M. haemomuris* genome copies per µl blood as a covariate in the linear models of component scores (see bottom panel in **Figure 36**) resulted in no significant differences in component scores for increased *M. haemomuris* genome copies for principal component 1 ($p=0.604$) and principal component 3 ($p=0.970$). Component scores for principal component 2 were significantly lower with increased *M. haemomuris* genome copies ($p=0.009$) and significantly lower for component scores for principal component 4 ($p=0.025$) that were due to lower component scores in the naïve group alone ($p<0.001$).

A visualisation of the component scores on all four principal components and normalised relative *M. haemomuris* genome copies per µl blood over the 14-day observation period is given in **Figure 36**.

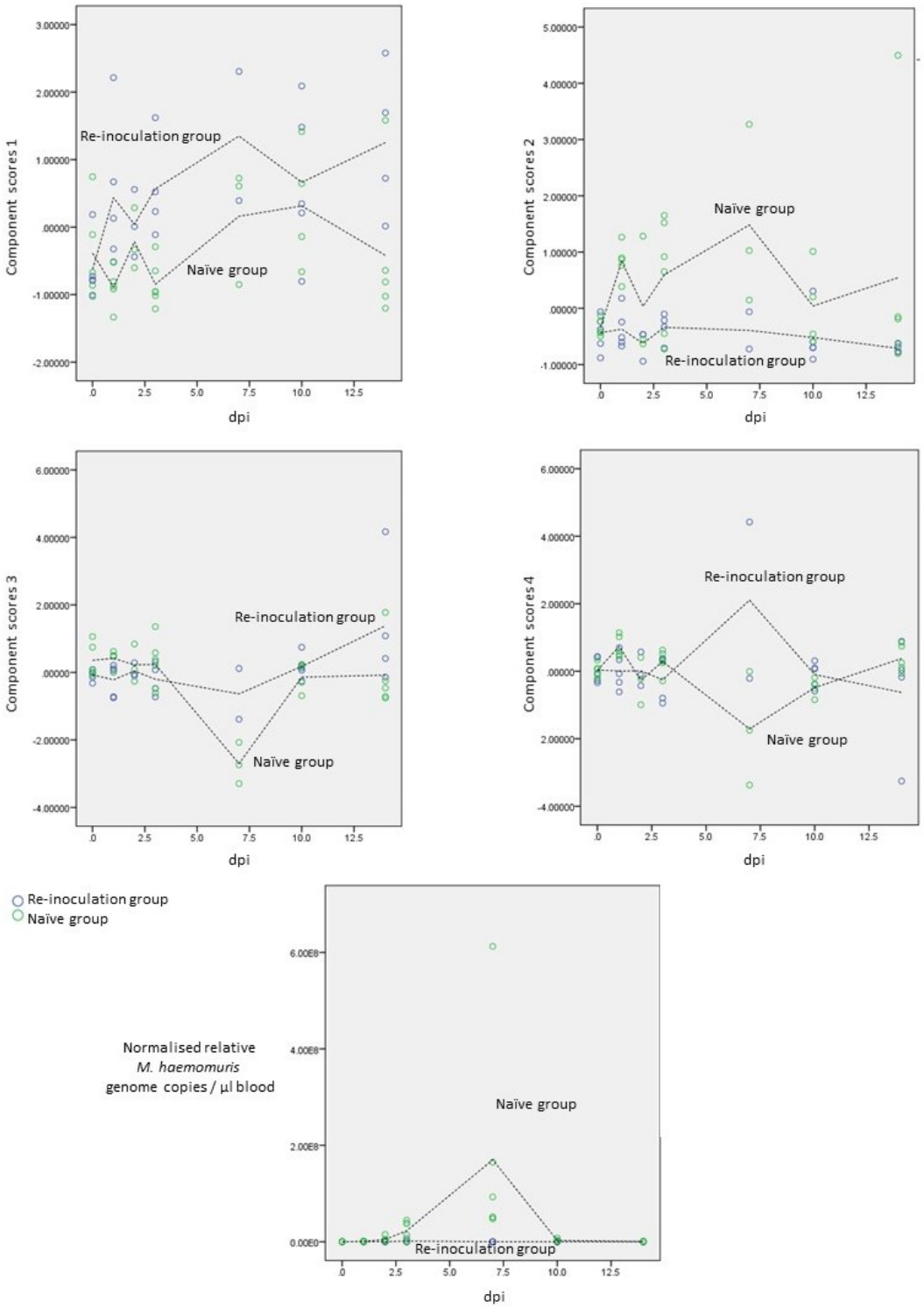


Figure 36 *Component scores (unrotated solution) spanning cytokine data of 14 consecutive days post inoculation (dpi) in Phase 2 of the cytokine expression experiment including a (linear) representation of normalised, relative M. haemomuris genome copies per µl blood over the same period. Calculated component scores for each of the four principal components (circles represent scores calculated for individual cytokine measurements) are plotted on the y-axis and dpi are plotted on the x-axis. Green circles represent component scores of the naïve group. Blue circles represent component scores for the re-inoculation group. Dotted black lines are linear interpolation lines (based on group medians) and labelled for both groups. Note, that component scores reflect cytokine concentrations but cannot be interpreted as such. Normalised relative M. haemomuris genome copies per µl blood have been added for comparison on a linear (not logarithmic) chart. Component scores 1, component scores on principal component 1; component scores 2, component scores on principal component 2; component scores 3, component scores on principal component 3; component scores 4, component scores 4 on principal component 4; Group, experimental group (naïve group versus re-inoculated group), Dpi, days post inoculation.*

The univariate analyses (**Table 45**) of component scores and experimental groups over time did not account for all variability within the dataset as observed in the 14-day observation period (see linear interpolation lines in **Figure 36**) especially variations of component scores around peak parasitaemia (7 dpi) for both experimental groups. The model was subsequently restricted to chronological changes (defining dpi as covariate to allow plotting of linear regression lines) and re-calculated for non-linear (quadratic) interactions but did not yield a better representation of the data apart from component 3, that was better described by a quadratic interaction of dpi and component scores with p=0.002. The univariate analyses of component scores in chronological order and allowing for quadratic interactions are presented in Appendix C, **Table A-C2** and **A-C3**.

4.3.4.2.4 Assessment of structure within the unrotated PCA dataset

Unrotated component loadings (reflecting the relationship between each datapoint and the extracted component) are optimised for maximal explanation of data variance but not necessarily structure within the dataset (see 4.2.5.4.2.4). Univariate analyses (**Table 45**) suggested principal components 1 and 2 should be able to identify a difference between groups. To visualise this relationship a scatterplot matrix was created, plotting component scores of all four principal components against each other. The scatterplot matrix of component scores is shown in **Figure 37**.

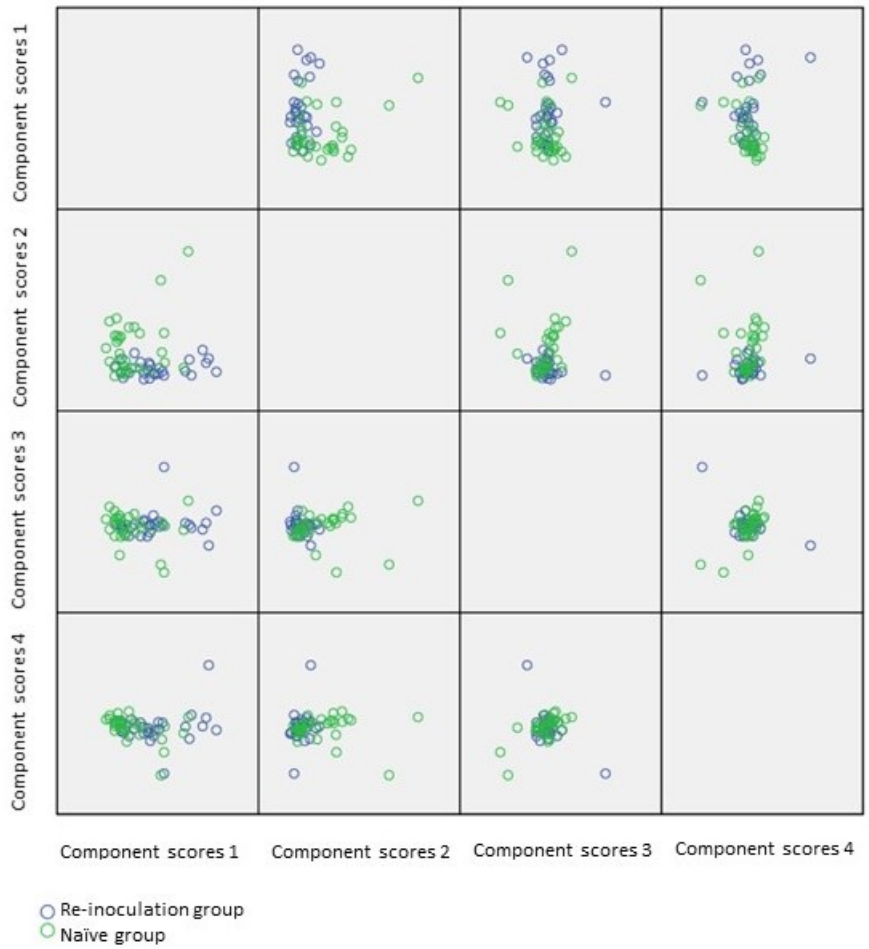


Figure 37 *Scatterplot matrix of unrotated component scores plotted against each other illustrating the relationships between the extracted components and clustering of the underlying dataset. As expected from univariate analyses (see* ***Table 45****), components 1 and 2 exhibited rough clustering that suggests a linear relationship between the two components. Green circles represent component scores of the naïve group and blue circles represent component scores for the re-inoculation group. Component scores 1, component scores on principal component 1; component scores 2, component scores on principal component 2; component scores 3, component scores on principal component 3; component scores 4, component scores 4 on principal component 4; Dpi, days post inoculation.*

Component scores on principal component 1 and 2 exhibited clustering, thus suggesting and roughly illustrating a linear relationship between the two components but without suggesting discriminative power to differentiate groups in two dimensions.

4.3.4.2.5 Consequences of component rotation on data structure and univariate analyses

To further analyse the data structure, component loadings were subject to Promax rotation which places the axes of principal components to maximise structure and allows for correlations between components as component axes are no longer required to be orthogonal to each other (see 4.2.5.4.2.4).

Solutions for the rotated principal component analysis are presented in Appendix C but will briefly be summarised here. Promax rotation of principal component loadings resulted in a change in cytokine composition loading on the extracted components. Rotated components will be referred to as 'rotated component 1' to 'rotated component 4' as they are not identical to the unrotated components described earlier in this chapter. After Promax rotation, 10 cytokines (IL-1b, IL-3, IL-4, IL-5, IL-6, GCSF, GMCSF, IFN-γ, MIP1b and TNF-α) were found to be loading on component 1, 4 cytokines (IL-9, IL-12p70, IL-13 and KC) loaded on component 2, 4 cytokines (IL-10, IL-12p40, MCP1 and MIP1a) loaded on component 3 and 5 cytokines (IL-1a, IL-2, IL-17, Eotaxin and RANTES) loaded on component 4 when using the same criteria for interpretation as described for the unrotated solution (4.3.4.2.2).

A revised component matrix is given in Appendix C, **Table A-C4**. The component scores on the rotated principal components were subject to univariate analyses as described for the rotated solution above (4.3.4.2.3) and summarised in Appendix C **Table A-C5**. Component scores for rotated components were significantly different for groups and dpi: Component scores for rotated component 1 were significantly higher in the re-inoculation group ($p<0.001$). Component scores for rotated component 2 were significantly higher in the re-inoculation group ($p<0.001$) compared to the naïve group and also significantly increased over time ($p=0.010$). There was a significant interaction between experimental groups and dpi ($p=0.002$), meaning that re-inoculated mice demonstrated a steep increase in component scores whilst naïve mice stayed low. Component scores on rotated component 3 were significantly different ($p=0.003$) over time but varied with group as the increase was more pronounced in the naïve group ($p=0.011$). Component scores on rotated component 4 were significantly higher in the naïve group ($p=0.019$) and did not significantly change over time for either experimental group ($p=0.981$).

Similar to the unrotated solution (4.3.4.2.3), allowing normalised relative *M. haemomuris* genome copies per µl blood as a covariate in the linear model resulted in significantly increased component scores with increased *M. haemomuris* genome copies ($p=0.019$) for the rotated component 3, reflecting a peak of scores over peak parasitaemia in the naïve group. A scatterplot matrix of the rotated solution is shown in Appendix C, **Figure A-C2**. As discriminative data clustering was not the aim of the current study and interpretability (based on immunological relationships between the cytokines) was not improved when using rotated component scores, the rotated solution to the PCA was not used to guide interpretation of PCA results.

4.3.4.3 Concentrations and univariate analyses of individual cytokines

Individual cytokine concentrations of the naïve and re-inoculated group mice, exsanguinated at 0, 1, 2, 3, 7, 10 and 14 dpi are given as absolute values (pg/ml) in **Figure 38** to **Figure 40** and presented as % change from baseline in Appendix C, **Figure A-C5**.

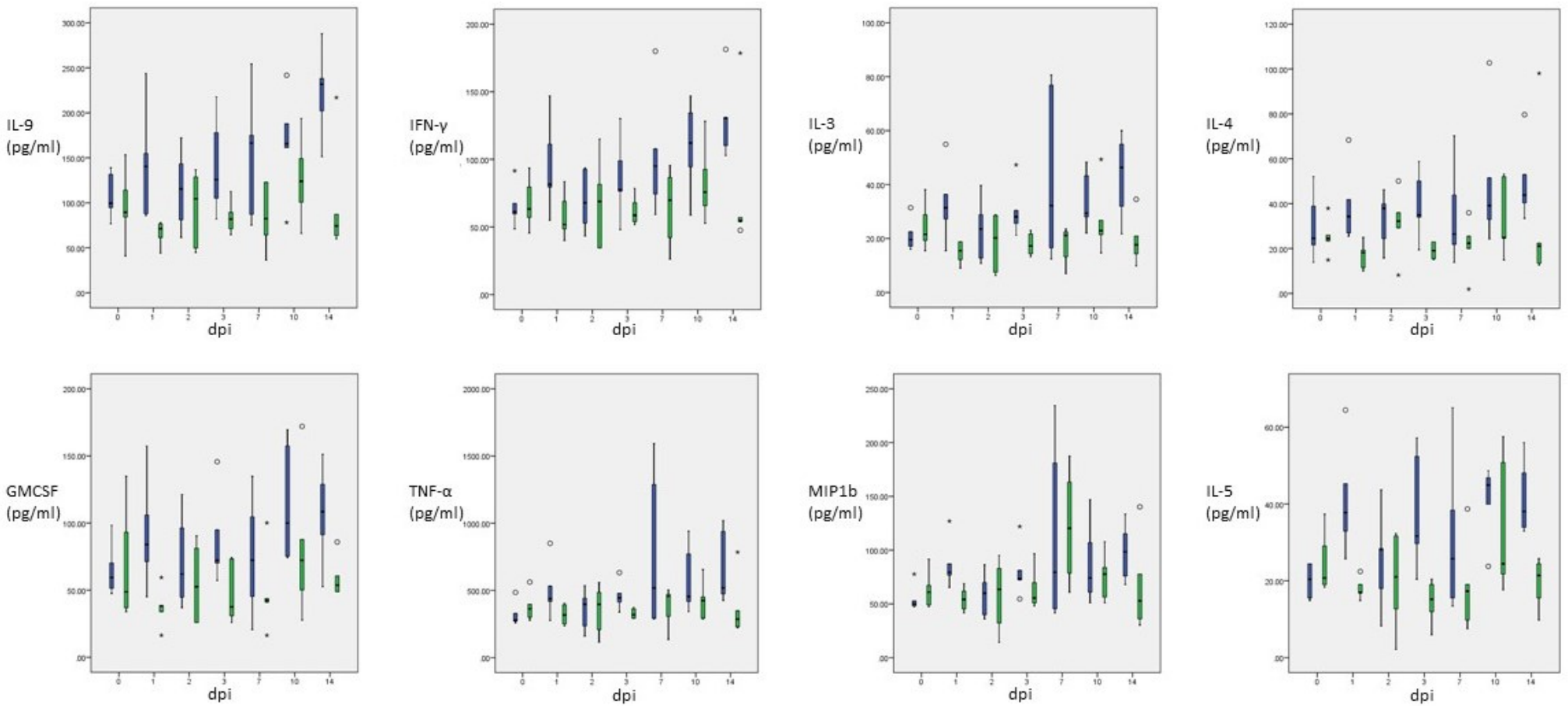


Figure 38 *Cytokines loading on component 1. Cytokine concentrations are plotted in absolute values (pg/ml) on the y-axis and days post inoculation (dpi) are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated as circles and dots. Experimental groups are indicated by the colouring of the bars: blue, re-inoculated group and green, naïve group. IL-3, Interleukin-3; IL-4, Interleukin-4; IL-5, Interleukin-5; IL-9, Interleukin-9; GMCSF, Granulocyte-Macrophage Colony Stimulating Factor; MIP1b, Macrophage Inflammatory Protein 1b; IFN-γ, Interferon Gamma; TNF-α Tumour Necrosis Factor Alpha.*

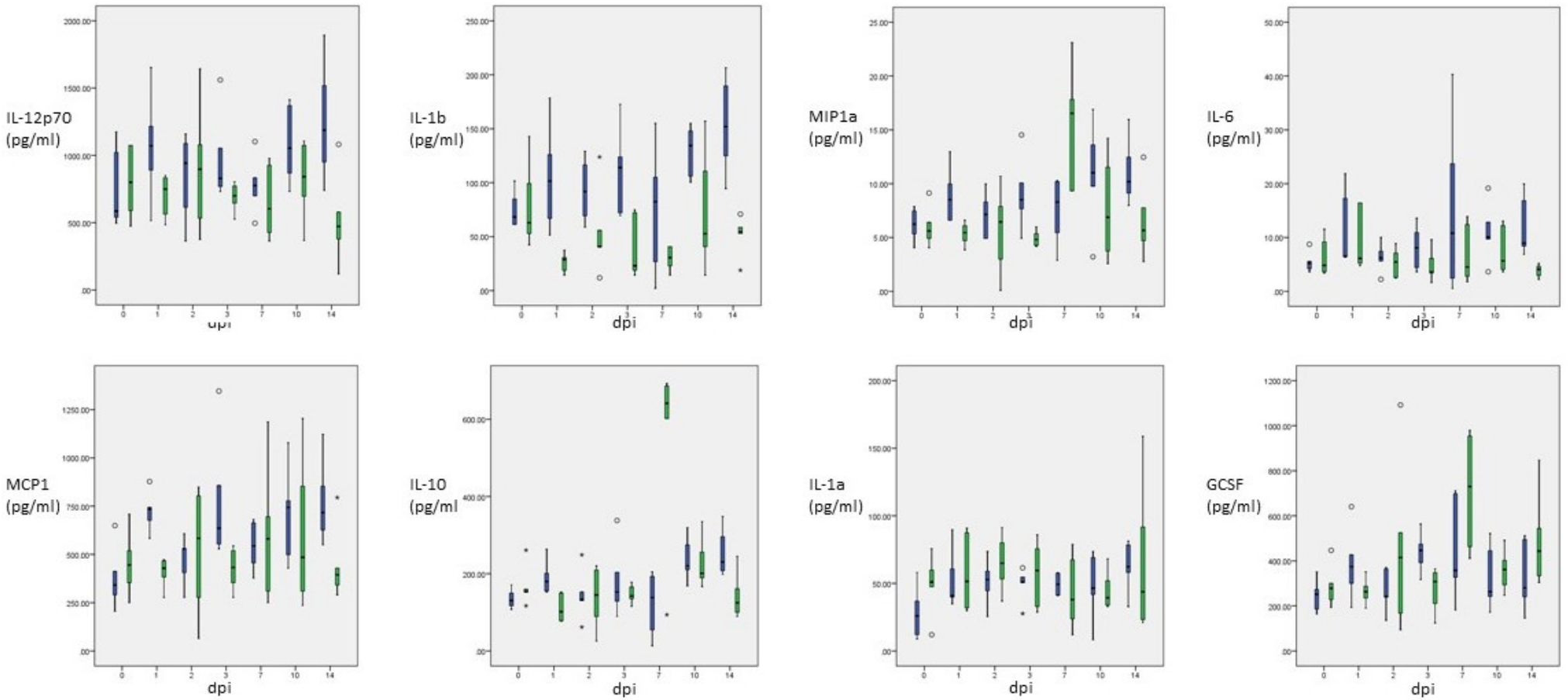


Figure 39 *Cytokines loading on component 1 (continued). Cytokine concentrations are plotted in absolute values (pg/ml) on the y-axis and days post inoculation (dpi) are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated as circles and dots. Experimental groups are indicated by the colouring of the bars: blue, re-inoculated group and green, naïve group. IL-1a, Interleukin-1a; IL-1b, Interleukin-1b; IL-6, Interleukin-6; IL-10, Interleukin-10; IL-12p70, Interleukin-12 Subunit p70; GCSF, Granulocyte Colony Stimulating Factor; MCP1, Mast Cell Protease 1 (CCL2); MIP1a, Macrophage Inflammatory Protein 1a.*

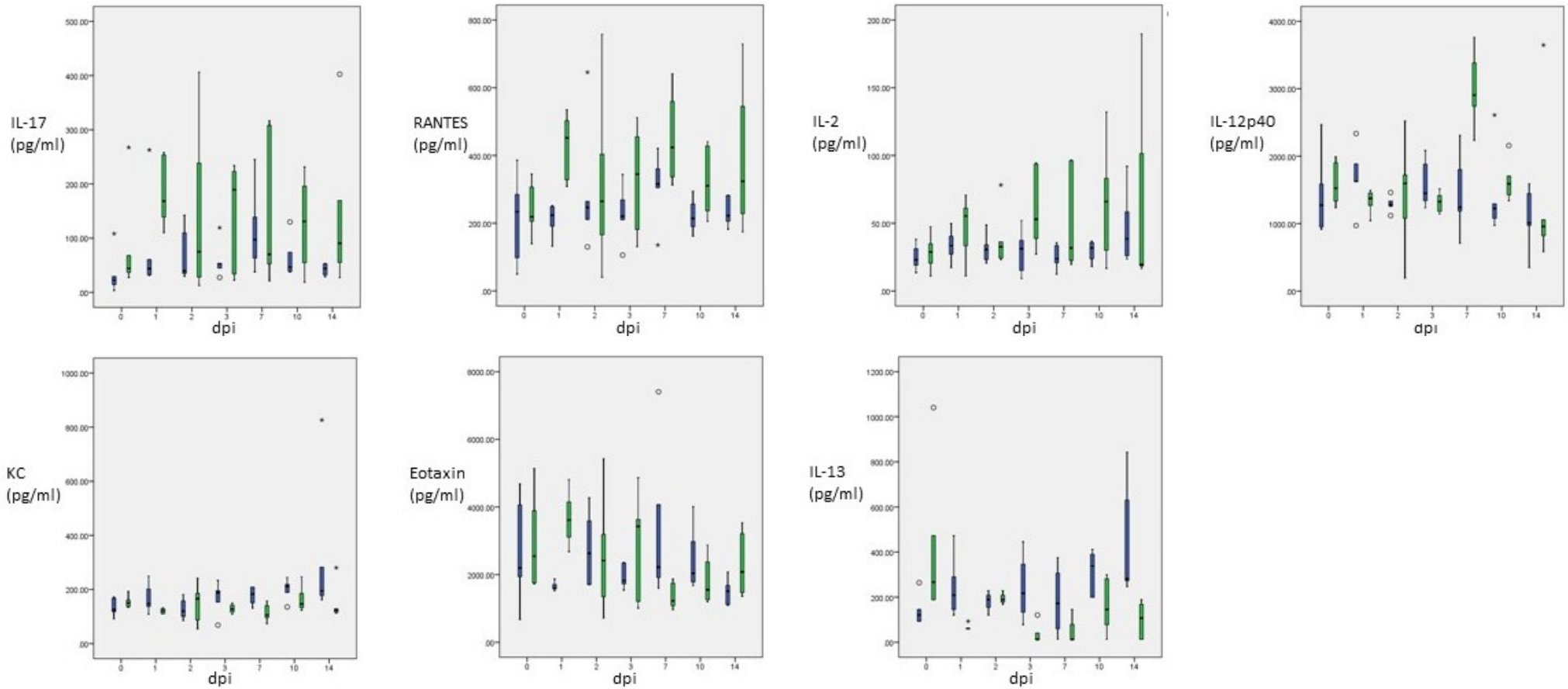


Figure 40 *Cytokines loading on Component 2 (top row) and 3 (KC, bottom row) as well as cytokines not significantly loading on any of the four principal components (Eotaxin and IL-13, bottom row). Cytokine concentrations are plotted in absolute values (pg/ml) on the y-axis and days post inoculation (dpi) are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated as circles and dots. Experimental groups are indicated by the colouring of the bars: blue, re-inoculated group and green, naïve group. IL-13, Interleukin-13; IL-17, Interleukin-17; KC, Keratinocyte Chemoattractant (CXCL1); RANTES, Chemokine 'Regulated on Activation, Normal T cell Expressed and Secreted', (CCL5), IL-12p40, Interleukin-12 Subunit p40; IL-1, Interleukin-1.*

As suggested by the linear model in the univariate analysis of component scores presented above in 4.3.4.2.3 and the loading of different groups of cytokines on the four principal components (**Table 44**), most cytokines loading on component 1 (**Figure 38** and **Figure 39**) showed a tendency towards higher and increasing cytokine concentrations in the re-inoculation group (e.g. IL-9, IFN-γ, IL-3, IL-4, GMCSF, TNF-α, IL-5, IL-12p70, IL-1b, IL-6 and MCP1). There was an apparent 'peak' at 7 dpi in the re-inoculation group for IL-3, TNF- α, MIP1b (including naïve group cytokine concentrations) and IL-6. A 'peak' appeared at 7 dpi in the naïve group for MIP1b (including re-inoculation group cytokine concentrations), MIP1a, GCSF and IL-10.

The cytokines, loading on component 2 (IL-17, RANTES, IL-2 and IL-12p40) demonstrated higher concentrations in the naïve group of the experiment, most pronounced at 7 dpi whilst cytokine concentrations in the re-inoculation group remained steady over the observation period.

Only KC was found to load on component 3 using the selected criteria and showed little variation, but a mild increase over time, in the re-inoculation group.

The cytokines not significantly loading on the four extracted components in the unrotated PCA interpretation showed a tendency to decrease over time from pre-inoculation levels (Eotaxin) and an increase over time in the re-inoculation group/decrease in the naïve group from pre-inoculation levels (IL-13).

General linear models for all cytokines, separated by groups, were calculated as well as isolated 'peaks' assessed for significant differences across groups and from pre-inoculation levels (data not shown). However, such analysis was not considered helpful in the interpretation of the current pilot study that aimed to identify a cytokine signature suggesting protection from re-infection and not individual cytokines over the course of infection, as will be discussed later.

4.4 DISCUSSION

4.4.1 Clinical signs and haematological changes during primary *M. haemomuris* infection

Due to the pilot nature of the haematology study, no statistical analysis was attempted. However, we have, for the first time, demonstrated the development of haematological changes including anaemia subsequent to *M. haemomuris* infection in C57BL/6 mice. The degree of anaemia roughly agreed with observations of *M. haemofelis* infection in cats (89) and extrapolating from feline data, some individuals could reach much lower (<10 % HCT) values than the observed range in our studies of 20.8-24.3 % HCT (see **Table 40**). Such individual lower responses could have been missed in our studies by sampling on a fixed set of days, but this limitation is not easily alleviated as repeated sampling from mice is likely to cause haematological disturbances on its own (366, 367). Another limitation of the haematology study is that there were no control mice to demonstrate a typical course of infection. However, as differences in inoculum dose were deemed negligible, very similar infection kinetics may be assumed.

4.4.2 Cycling of *M. haemomuris* genome copies per µl blood and peracute death of mice

Cycling of *M. haemomuris* genome copies over the course of infection has not previously been observed but is a feature of *M. haemofelis* infection (89). Interestingly, the cycling of *M. haemomuris* genome copies appeared at the same number of passages from the original *Apodemus* spp. isolate as did more frequently occurring peracute deaths at peak parasitaemia. Fatal outcomes following primary *M. haemomuris* infection had been observed (data not shown) previously but were isolated rare events and attributed to secondary complications such as lymphatic neoplasia in old animals or excessive bleeding from the sampling site. This raises the question of whether obtaining inocula during exponential expansion of *M. haemomuris* increases pathogenicity and whether serial passaging of our isolate had a pathogenicity-enhancing effect. Although typical infection kinetics have now been described for 14 days following naïve infection with *M. haemomuris*, future studies should include daily sampling on larger groups of mice to further elucidate the effect of cycling genome copies and a potential correlation with more severe outcomes. The current dataset does not include enough cases (5 mice with observed cycling and 4 peracute deaths, one mouse that died following cycling genome copies) to either support or refute this hypothesis.

4.4.3 Principal component analysis suggests Th17-driven immune response in naïve *M. haemomuris* infection

We have successfully extracted four principal components to identify the cytokine expression signatures following naïve inoculation with *M. haemomuris* and re-inoculation (akin to protection from re-infection) with the same organism. The first two components were considered meaningful in analysis although different ways of clustering the dataset (i.e. Promax rotation as briefly summarised in 4.3.4.2.5) or employing different criteria of defining meaningful component loading (see 4.3.4.2.2) were left underexplored.

Principal component 1 comprised a pleiotropic immune response consisting of Th1 (i.e. IFN-γ, TNF-α and IL-12p70), Th2 (i.e. IL-4, IL-5, IL-9 and IL-13) and Th17 (i.e. IL-6, GMCSF) components as well as various chemokines activating these pathways (i.e. MIP1a, MIP1b, IL-1a and IL-1b). These cytokines were found to be expressed more in the re-inoculation group when compared to the naïve group and subject to an increase over time as shown in **Figure 38** and **Figure 39** and reflected in component scores on principal component 1 (see **Figure 36**, **top left panel**).

Looking at individual cytokines loading on component 1 (**Figure 38** and **Figure 39**), the observed 'peaks' at 7 dpi occurred in the re-inoculation group for the proinflammatory cytokines TNF-α and IL-6 that act as endogenous pyrogens (368) and IL-3 that mediates allergic and eosinophilic inflammation (369) as well as the chemokine MIP1b that mediates granulocytic inflammation by further inducing TNF-α, IL-6 and other proinflammatory mediators (370). Both Th1 and Th2 pathways appear to be active during protection from re-infection with *M. haemomuris*, which includes the cytokine 'peak' seen at 7 dpi for IL-3, TNF-α and MIP1b that did not have a corresponding peak in *M. haemomuris* genome copies per µl blood in the re-inoculation group. However, these cytokine peaks did correspond to peak bacteraemia in the naïve group at the same time after inoculation (see **Figure 32** and **Figure 35**). Interleukin-6, IL-3 and TNF- α are all commonly associated with chronic inflammatory disease (369, 371, 372), however these cytokines did not remain elevated throughout the study period, suggesting successful dampening of pro-inflammatory stimuli after controlling excessive *M. haemomuris* proliferation. The 'peak' in cytokine concentrations as seen in cytokines loading on principal component 1 (**Figure 38** and **Figure 39**) was also present in the naïve group but occurred in IL-10, GCSF and MIP1b (that also increased in the re-inoculation group). Whilst MIP1b as mentioned above and GCSF have primarily proinflammatory roles, especially GCSF that drives neutrophilic inflammation and potentially aggravates tissue destruction (373), IL-10 has evolved to regulate inflammation and protect from excessive tissue damage, especially in allergic and auto-immune disease (374). This increase of IL-10 at peak bacteraemia agrees with current therapeutic strategies in haemoplasmosis in cats that do not involve corticosteroids unless in rare, extreme cases, although translatability of these mouse model findings to feline haemoplasmosis has not yet been assessed. Unfortunately, no cytokine data were available from the mice that died at the time of peak bacteraemia (4.3.1.2) to correspond potential dysregulation of inflammation to mortality. The increase of IL-6 and TNF-α that was more pronounced in the re-inoculated group and the peak of IL-10 coinciding with peak bacteraemia agrees with previous findings in cytokine mRNA levels in response to infection/re-infection with *M. haemofelis* (107).

Principal component 2 indicated the presence of Th17 pathway cytokines (IL-17, IL12p40 and IL-2), that are thought to clear extracellular pathogens not effectively handled by Th1 and Th2 pathways (375). The component scores on component 2 were found to significantly differentiate between the naïve and re-inoculated groups, especially at the time of peak bacteraemia at 7 dpi (**Figure 36, top right panel**).

The difference in component scores on principal component 2 between experimental groups was reflecting rising and elevated plasma cytokine concentrations in the naïve group (**Figure 40, top row**). Interleukin-17 is the hallmark of the pro-inflammatory Th17 response as discussed above in 4.1.1.1.3. Interleukin-12p40 is a subunit of IL-23 (shared between IL-12 and IL-23) that is involved in maintaining a Th17 response (376). Interleukin-12 however, has a different function, and drives a Th1 response and secretion of IFN-γ, which inhibits Th17 proliferation (377). As IL-23 was not measured in the current study, confirmatory measurement of IL-23 would be required to rule out other sources of IL-12p40 in primary *M. haemomuris* infection. Interleukin-2 and IL-1b convert T_{regs} into Th17 cells, promoting an inflammatory response (378). RANTES is a granulocyte and lymphocyte chemoattractant, associated with inflammatory disease and autoimmune disorders but also plays a vital role in immune response to viral infection (379). In contrast to other pro-inflammatory chemokines, IL-17 seems to have a suppressive effect on RANTES in most environments, limiting further lymphocyte infiltration (380) whilst Th17 cells themselves are strongly attracted by RANTES to sites of inflammation (381). Receptors for RANTES are also expressed on NK-cells and play a role

in homing NK cells and Th17 cells to the red pulp of the spleen (382); RANTES has been shown to promote resistance to infection with *Leishmania major* (383).

The differential clustering of the cytokines on principal components 2 and 1 (see **Figure 37**) suggests that Th17 pathways play a vital role in naïve *M. haemomuris* infection but not in protection from-reinfection, as the re-inoculation group exhibited higher concentrations of cytokines associated with Th1, Th2 and Th17 pathways. A subjective favouring of Th1 cytokines (i.e. IFN-γ, TNF-α, as shown in **Figure 38**) early in the re-inoculation group would have to be explored further in more targeted studies. Cytokine ratios, that have traditionally been used to define cytokine expression patterns in infections, allergic diseases (384, 385) or haemoplasma infection (107) were not explored in the current study in favour of the PCA. Further studies could consolidate our current findings and design experiments to look at a limited number of cytokines, their progression over time and try to identify risk factors for more severe clinical disease or lack of protection by the use of higher mouse numbers in pre-defined experimental settings.

Measuring other Th17-type cytokines not included in the current assay (e.g. IL-21 or IL-22) would help consolidate the hypothesis that Th17 cells play a vital role in primary infection with *M. haemomuris*. For instance IL-22 is another effector cytokine produced by Th17 cells and regulates epithelial barrier function during infection but can also promote autoimmunity (337). Interleukin-21 is also produced by Th17 cells and amplifies Th17 responses in an autocrine loop, hence favouring Th17 over T_{reg} differentiation (337).

Principal component 3 was found to mainly represent KC as found in wound healing and other cytokines (like IL-13, although it was not included in the analysis due to its low component loading) that exhibited a decrease at peak parasitaemia in the naïve group and an increase in early recovery in the re-infected group (**Figure 40, bottom row**). As a result, this component was able to differentiate between the groups as an interaction with dpi (see 4.3.4.2.3). The chemokine KC has been shown to be critical for neutrophil migration and clearing of septicaemia (386), and appears to share regulatory pathways with IL-17 (387, 388). Interleukin-13 is a Th2 type cytokine, playing a role in chronic infection, allergic airway disease and is associated with protective immunity against nematodes (389).

Principal component 4 was found to be less meaningful than the previous components but was retained for analysis as an additional dimension. Similar to principal component 3, it appeared influenced by cytokines/chemokines that had a distinct but less pronounced 'peak' in one of the groups over the course of infection (**Figure 40, bottom row**), however further analysis was not attempted.

When assessing component scores over time (**Table 45**), the observed changes in component scores were primarily analysed as a general linear model and not restricted to chronological order (as the variable of dpi was entered as a fixed factor rather than a covariate into the univariate analyses allowing for 6 degrees of freedom). Hence, the statistical significances listed in **Table 45** only refer to any changes over time but not the exact development of those changes. A better fitted model could not be achieved by restricting the degrees of freedom (equivalent to ordering the dpi chronologically by rendering dpi as a covariate) and allowing for non-linear (i.e. quadratic) interactions. The lack of significant differences described by a quadratic curve over time could have been caused by the scattering of the data and the observed 'peaks' at 7 dpi that were not preceded by gradual increases or decreases over time. The significance of the 'peaks' at 7 dpi was already assessed in the linear model summarised in **Table 45**. It was, however, not the purpose of this study to fit a perfect model of component scores over time but to describe a broader level of variability and clustering within the

dataset. Hence no linear regression lines were plotted in **Figure 36** as they could be interpreted for significance (**Table 45**) and dotted interpolation lines are given to point towards potential non-linear relationships that could guide further studies and cytokine changes of interest over the course of infection with *M. haemomuris*.

Changes in cytokine concentrations over time (**Figure 38** to **Figure 40**) were presented as box plots without further statistical analyses. Such analyses (e.g. general linear models to look at time courses or Mann-Whitney-U statistics to compare individual days between groups) were performed but were not presented within this chapter as such "cherry picking" would undoubtedly have produced a high number of significant results without adding to the overall interpretability of the findings. Additionally, in the current pilot study, the effect size was undoubtedly close to individual variation, hence selecting out individual 'peaks' between days and groups would not have been appropriate without defining a biologically meaningful question and addressing it further with a suitably powered study.

4.4.4 Limitations of the current mouse model and further work

The studies described in this chapter have successfully demonstrated the development of haemoplasmosis in C57BL/6 mice after infection with *M. haemomuris* based on haematology, splenomegaly, bacteraemia-associated deaths and an inflammatory cytokine response over 14 days following primary infection. Although the described haematology- and cytokine expression studies were pilot in nature, the newly established mouse model can now be used to investigate clinical and laboratory changes that occur in host animals infected with haemoplasmas. The use of the mouse model is not limited by focusing on infection kinetics alone. Full validation for subsequent research purposes will still require further studies to define confidence intervals for defined changes before further conclusions can be drawn. The translatability of findings from mouse to cats or other host species has not been determined for haematology, splenomegaly, risk of death or cytokine expression. However, we now have a surrogate mouse model of haemoplasma infection and haemoplasmosis that allows for the design of more targeted studies. We have also demonstrated that the use of amplifier mice (4.2.1.5.2 and 4.2.1.6.2) appears to eliminate the problem of loss of inoculum viability that was seen in the work described previously in Chapter 3.

In the cytokine expression study, cytokine levels were measured in plasma. Whilst their role is to recruit cells of the immune system to the site of infection (blood), they typically act on their paracrine environment, only reaching cells in immediate proximity (322). The spleen is the specialist organ that usually deals with blood borne pathogens and as such, could be more likely to pick up paracrine cytokine signals (322). Whilst spleens from all mice exsanguinated as part of the cytokine expression study and haematology study were cryopreserved at -80°C, splenic cytokine expression was not addressed by our methods, potentially missing detection of a more polarised immune response over the course of infection. Whilst splenic cell suspensions might not be comparable to plasma cytokine expression, due to required sample preparation and tissue lysis (346), future experiments could involve measuring splenic cytokine expression *in vitro*, with or without prior extraction of lymphocyte populations.

A statistical limitation of the cytokine expression study that has not yet been mentioned for the plasma cytokine measurements discussed above, is the possible introduction of a type-1 error (rejection of a true null hypothesis or 'false positive' finding). Although the naïve and re-inoculation groups and their respective control groups (see **Figure 27**) were matched in age and procedures, some cytokines could still reflect an effect on the subgroups exsanguinated on a given day, or at least be influenced by it. As the precision and repeatability of the assay under the employed

conditions was found to be acceptable (see 'evaluation of quantitation', 4.3.4.1) we did not run our samples in duplicate or triplicate due to the financial considerations of a pilot study. It is unlikely, but possible, that some of the scattering of absolute cytokine measurements (**Figure 38** to **Figure 40**) was due to assay variation rather than individual variation within the experimental groups and would have been greatly reduced if sufficient replicate measurements were used.

The interpretation of the reduced dimensions of a dataset obtained through PCA is inherently subjective as it is based on the current understanding of biological phenomena that might be subject to change over time. Even though PCA is one of the most commonly used methods to reduce data dimensions and extract features in the medical field, as it plots the variability of the data on a lower number of axes (components), it can introduce discriminatory features that lack biological meaning (390). This is especially true for rotation of components to maximise discriminatory power at the cost of displaying the maximum variability within a dataset (391), which is why further rotations or other constraints, such as making a component sparse (392), were not explored in the current study. Future studies should concentrate on asking more defined questions (i.e. better defining the IL-17/IL-23 axis or following IFN-γ and TNF-α over the recovery time, prior to re-inoculation) in pre-defined environments and use the current dataset as guidance rather than directly inform decision on preventative strategies. Such strategies could ultimately identify predictors of more severe outcomes or less effective development of protective immunity, which in turn will guide development of vaccines and vaccine adjuvants to ensure an adequate immune response (319).

5 *IN VITRO* CULTIVATION OF *MYCOPLASMA HAEMOMURIS*

5.1 INTRODUCTION

5.1.1 Haemoplasma whole genome sequence data and metabolic capacities

Haemoplasmas are currently unculturable *in vitro* (11). Due to their narrow biological niche in the bloodstream of their hosts, they have likely undergone metabolic reduction further than other *Mycoplasma* species (72). The lack of certain metabolic core enzymes and the presence of a variety of transporter proteins is an indication that haemoplasmas are fully equipped to scavenge vital substrates off their hosts and utilise nutrients they find in abundance within the bloodstream (72). Similar to the erythrocytes they adhere to, haemoplasmas use glycolysis as their primary source of energy, but in contrast to erythrocytes they have lost their pentose-phosphate pathway that generates NADPH to prevent oxidative stress, and require importation of nicotinamide for NADPH generation (72, 196). Having lost the ability to synthesise purine and pyrimidine nucleotides (substrates for RNA and DNA synthesis), these must also be imported alongside nine essential vitamins and other substrates to enable haemoplasmas to replicate (393). The feline haemoplasma, *M. haemofelis,* requires hypoxanthine, adenine, guanine, uracil and cytidine monophosphate to maintain its nucleotide metabolism, based on whole genome sequence analysis (393), but the concentrations required and any potentially inhibitory effects cannot be directly inferred from genomic data. Hypoxanthine is a by-product of purine nucleotide metabolism in erythrocytes, and is also required for growth of other pathogens inhabiting a similar biological niche, such as *Plasmodium falciparum* (394). Hypoxanthine is also released from erythrocytes, particularly during oxidative stress as expected during haemoplasmosis and suboptimal *in vitro* cultivation conditions (395). Adenine is a purine nucleobase required for the growth and spore formation of *Clostridium* spp. and *Bacillus* spp., which are phylogenetically close to *Mycoplasma* spp. (396, 397). Other purine nucleobases, such as guanine or the pyrimidine derivatives cytosine and cytosine monophosphate, have similar effects on *Bacillus* spp. growth when in high concentrations, although they can sometimes have inhibitory growth effects (398). The uptake of nucleotides and their precursors has been demonstrated for several *Mollicutes* (399, 400) but they are generally less studied in their metabolic activities than other *Tenericutes* due to their fastidious nature and difficulties in culturing them.

5.1.2 Recent attempts of *in vitro* cultivation using data from whole genome sequencing

Recent attempts to cultivate *M. suis* outside of the porcine host, informed by whole genome sequence data, resulted in nanotransformation, but not specific growth in the mycoplasma media SP-4 and Hayflick's medium (242). Nanotransformation represents organisms with a reduced size, which are putatively metabolically inactive, and can be detected and differentiated from their normal state by electron microscopy. Quantification by qPCR of the nanotransformed organisms showed a stationary level of genome copies per ml of medium over two to three weeks of *in vitro* cultivation in media supplemented with haemin, haemoglobin and transferrin, but not in media devoid of these iron-binding proteins where drastic decreases in copies per ml occurred (242). Unfortunately in the latter study, Schreiner, Hoelzle (242) could not assess the viability of the nanotransformed *M. suis*, so the nature of the nanotransformed organisms that resulted from this growth experiment is unknown.

More recently, Hicks (216) attempted to cultivate *M. haemofelis* outside of the feline host and produced promising, but not reproducible, results when supplementing MEXp (243) medium with 120µM hypoxanthine over 4 days. Unfortunately, due to patent restrictions, the exact composition

of the medium could not be disclosed. Inoculation of other media, such as RPMI-1640, produced fluctuations of *M. haemofelis* genome copies per ml over the first hour post inoculation (which was not assessed for the MEXp studies), but did not result in sustained growth when incubated for a longer time period (216). Cultivation with Dulbecco's modified Eagle's medium (DMEM) or co-cultivation with cell lines such as Crandell Rees feline kidney (CRFK) or the armyworm ovarian cell line SF-9 also did not result in *M. haemofelis* growth (216).

5.1.3 Rationale of using intact cells in cultivation experiments

Very early observations of *E. coccoides* by light microscopy (203, 238) reported them to exist in a form that was not attached to erythrocytes. The porcine haemoplasma *M. suis* was also visualised 'free' on blood smears as part of studies of erythrocyte-membrane alterations during *M. suis* infection (116). However, haemoplasmas have not generally been found 'cell-free' in the plasma fraction (that is not associated with erythrocytes) from infected animals nor in tissues (11, 99, 285) by molecular techniques. Additionally, in recent experiments cultivating *M. suis* (241, 242) and *M. haemofelis* (216), no sign of free growth following erythrocyte detachment of the haemoplasmas was detected. Thus, these studies suggested that future cultivation studies may benefit from the use of intact erythrocytes. Erythrocytes have a limited life-span of about 40 days in mice (401) and are not the only erythroid cells found in the mammalian/murine bloodstream (402), so additionally the use of erythroid progenitor cells seemed logical to use in long-term *in vitro* cultivation experiments. Furthermore, the only reports of *in vitro* propagation of a haemoplasma species, *E. coccoides*, used chicken embryos (9), and the yolk sac is a source of erythroid progenitor cells in chicken, murine and human embryological development (403). Immortalised erythroid progenitor cell lines are not available for cats or pigs, but their murine counterparts have played an important role in the development of laboratory-grown blood transfusions (404), and are available for use in culture experiments.

5.1.4 Rationale for using amplifier mice to generate viable *M. haemomuris*

The studies by Nonaka, Thacker (241) and Hicks (216) that failed to produce significant haemoplasma growth, used cryopreserved blood (containing polyvinylpyrrolidone, glycerol or DMSO). Cryopreservation could have affected haemoplasma viability, and viability may also have been affected by prolonged storage in liquid nitrogen or at -80°C. A possible negative effect of cryopreservation on haemoplasma viability is supported by our failed infection attempts in Chapter 3; 3.3.2.2, using cryopreserved blood samples. Additionally, cryopreserved samples used by Hicks (216) to inoculate cats according to the low-dose *M. haemofelis* infection model described by Baumann, Novacco (189) were not effective. By having a rapidly available source of viable *M. haemomuris* organisms through our recently established mouse model, we were given the opportunity to perform cultivation experiments using freshly sourced, and thus likely viable, haemoplasmas in a reasonably cost-effective way.

5.1.5 Rationale for using sentinel mice to detect *M. haemomuris* viability

As discussed above in 5.1.2, haemoplasma viability could not be assessed in previous cultivation attempts likely due to costs and ethical concerns of housing host animals for the sole purpose of infecting them with cultivated haemoplasmas to assess viability. However, failure to do this viability confirmation conflicts with Koch's third postulate, which states that the disease must be reproduced when a specific bacteria is re-introduced into a susceptible host (405). Using live animals as a binary assay (infected vs. uninfected) to detect haemoplasmas has been suggested to identify more or less favourable haemoplasma culture conditions, but has not been used in subsequent studies (242). A mouse model would enable us to assess viability, although ethical concerns with the use of live animals remain. The mouse model would allow inoculation of sentinel mice to indicate the presence

of viable haemoplasma organisms (*M. haemomuris*) in cultivation attempts independently of *in vitro* maintenance or growth.

5.1.6 Objectives

The aim of this study was to define the maintenance and growth requirements for the haemoplasma species *M. haemomuris* using the mouse model of infection to assess viability after *in vitro* propagation. The growth of *M. haemomuris* in liquid culture was studied, with culture conditions optimised to support growth of murine erythroblast progenitor cells and their differentiation into mature erythroblasts, to provide an alternative cellular substrate to erythrocytes. Essential metabolic substrates not usually included in the base medium composition, such as hypoxanthine or nucleotide precursors, were added to try and define media compositions that were favourable for *M. haemomuris* viability and growth. The viability of cultivated *M. haemomuris* was defined by the inoculation of naïve sentinel mice with cultivated *M. haemomuris,* using them as a binary detection mechanism where successfully infected mice detect viable *M. haemomuris* organisms compared to not successfully infected mice detecting non-viable *M. haemomuris,* respectively. Under *in vitro* conditions, *M. haemomuris* growth was detected by comparison of repeated samples from *in vitro* cultures followed by DNA extraction and qPCR for *M. haemomuris*.

5.2 MATERIALS AND METHODS

5.2.1 Assessment of *M. haemomuris* assay variability

In order to reliably differentiate *M. haemomuris* growth during *in vitro* cultivation studies from qPCR data, the intra-assay and inter-assay variations of the *M. haemomuris*-specific assay (3.3.1.4) needed to be determined.

5.2.1.1 Origin of samples

Dilutions of PCR amplicons, spanning the qPCR assay's target sequence (see 3.3.1.4) were prepared in mouse gDNA. Background gDNA was sourced from *M. haemomuris*-negative mice during pre-infection screening from earlier studies as previously described in 4.2.1.3. Extracted DNA samples were stored at -20°C until use.

5.2.1.2 Intra-assay and inter-assay variation determination

A dilution series of four sequential ten-fold dilutions was prepared, and each dilution was run in ten replicates on the same qPCR plate to determine intra-assay variation for each dilution. Ten threshold cycle values and ten calculated relative *M. haemomuris* genome copies per reaction (calculated as in 2.2.1.2.2.2.2) were recorded for each dilution. The dilution series was stored at 4°C over-night and the experiment repeated the next day with freshly prepared qPCR reagents to determine inter-assay variation. All reactions were carried out on the same thermal cycler (Mx3005P; Agilent) and plates assembled by manual pipetting rather than pre-mixing of master mix and DNA template to mimic actual experimental conditions to be used in the cultivation experiments.

5.2.1.3 Calculation of coefficients of variation

Results of the intra-assay and inter-assay variation studies were expressed as means and standard deviations (stdev) of all replicates for each dilution on each qPCR plate on both experimental days. Means and stdevs were calculated for threshold cycle values and relative *M. haemomuris* genome copies per reaction. Coefficients of variation (%CV) were calculated using Excel (Microsoft® 2013). The intra-assay % CV was calculated for each dilution as (stdev/mean)*100 using values from replicates obtained on the first day. The inter-assay %CV was calculated as (stdev/mean)*100 using values from all 20 replicates per dilution obtained on both days.

5.2.2 *In vitro* cultivation studies

5.2.2.1 Animals

All mice were female, SPF C57BL/6 and sourced from Harlan Laboratories UK Ltd., Loughborough, UK.

5.2.2.1.1 Ethical approval

All studies involving laboratory rodents were subject to ethical review (Animal Welfare Ethical Review Body of the University of Bristol) and covered by a Home Office licence (PPL PCFCBE2EB).

5.2.2.1.2 Housing conditions

Mice were housed under conditions and monitored as previously described in 2.2.3.2 but separated into individual cages after inoculation.

5.2.2.1.3 Screening mice for pre-existing *M. haemomuris*-infection

Mice were allowed an acclimatisation period of three to ten days. One week prior to all experiments, mice were uniquely identified, and had a 10µl blood sample withdrawn for generic haemoplasma qPCR analysis as outlined in 2.2.3.3.

5.2.2.1.4 Amplifier mice to generate viable inocula for *in vitro* cultivation studies

Due to concerns over cryopreservation of *M. haemomuris,* amplifier mice were used to generate viable *M. haemomuris* inocula to inoculate culture media preparations for *in vitro* culture experiments.

5.2.2.1.4.1 Inoculation and monitoring of infection in amplifier mice

Inoculation procedures for the studies described within this chapter did not vary from the procedure described in 2.2.3.4.1. Infection with *M. haemomuris* was monitored by daily blood sampling of 10 µl followed by DNA extraction from the blood sample and *M. haemomuris*-specific qPCR as described previously in *3.2.2.3.2.*

5.2.2.1.4.2 Collection of M. haemomuris-infected blood from amplifier mice

Collection of blood for inoculation of culture media preparations was performed using the technique outlined in 2.2.3.7. However, no cryopreservative was added to the blood at any point and aseptically collected samples were transported to the laboratory for use in cultivation experiments within 5 minutes of collection under isothermal conditions.

5.2.2.1.5 Sentinel mice to monitor *M. haemomuris* viability after *in vitro* cultivation experiments

5.2.2.1.5.1 Inoculation of sentinel mice with M. haemomuris after in vitro propagation

As specified in the individual experiments, aliquots of *M. haemomuris* in culture media suspension were inoculated into 9 to 15-week-old mice using the technique described in 2.2.3.4.1. Total inoculation volume was 200µl per mouse, and inocula were prepared by allowing the cultured cells (typically 500µl) to sediment, removing the supernatant, and resuspending the cells in 200µl of fresh culture medium of the previously used composition to generate an inoculum. The total volume of culture wells (typically 500µl) could not be used for inoculation as this would have been too great a volume to inoculate intra-peritoneally into each mouse (as stipulated by regulated procedures covered by the Home Office licence).

5.2.2.1.5.2 Monitoring of sentinel mice after inoculation with in vitro propagated M. haemomuris

Infection with *M. haemomuris* was monitored by daily blood sampling of 10µl followed by DNA extraction from the blood sample and *M. haemomuris*-specific qPCR as described previously in

3.2.2.3.2. Mice were monitored for 16 consecutive days following inoculation unless specified otherwise.

5.2.2.2 Preparation of culture media and stock additives

5.2.2.2.1 Preparation of StemPro™-34 culture medium

StemPro™-34 serum-free stem cell medium (Life Technologies, Gibco, UK) was mixed with frozen supplement (StemPro™-34 Nutrient Supplement) prior to use and stored at 4°C. After six months of use (duration of experiment was longer than anticipated due to overlap with the studies described in Chapter 4) culture medium was discarded and replaced by a freshly prepared batch. StemPro™-34 is a specialist medium optimised to support the growth of human haematopoietic stem cells and was chosen as a base medium to try and co-cultivate murine erythroblast progenitor cells (stem cells) as described by von Lindern, Deiner (406) with *M. haemomuris*.

5.2.2.2.2 Preparation of hypoxanthine

A stock solution of hypoxanthine (Gibco) of 0.1M was prepared in 0.1N NaOH and filtered through a sterile 0.2µm filter (Nalgene, Thermo Fisher, UK) and stored at 4°C until use.

5.2.2.2.3 Preparation of $CaCl_2$ and $MgCl_2$

To determine if $CaCl_2$ or $MgCl_2$ concentrations in the base media, optimised to grow non-adherent cell lines, required adjustment to mirror mouse plasma levels and favour adherence of cells, the concentrations of ionised calcium and magnesium in StemPro™-34 were increased by 25%; adding 41µl/ml $CaCl_2$ and 25µl/ml $MgCl_2$. Stock solutions of $CaCl_2$ and $MgCl_2$ (10mg/ml) were prepared from anhydrous electrolyte preparations (Gibco) and filtered through a sterile 0.2µm filter (Nalgene) and stored at 4°C until use. Electrolytes are recognised co-factors in cell-adhesion and may play a role in pathogen adhesion (407, 408).

5.2.2.2.4 Preparation of nucleobases, nucleosides and nucleotides

Mycoplasma haemofelis was used as an example of a phylogenetically related haemoplasma that has full genome sequencing data available for exploration of metabolic capacities for use in the cultivation studies of *M. haemomuris*.

Stock solutions of 20mg/ml were prepared as follows:
Nucleobases: Adenine (Sigma) was dissolved in 0.5M HCl, Guanine (Sigma) was dissolved in 1N NaOH, Thymine (Sigma) was dissolved in 0.5N NaOH, Uracil (Sigma) was dissolved in 0.5N NaOH, Cytosine (Sigma) was dissolved in 0.5M HCl.
Nucleosides: Cytidine (Sigma) was dissolved in distilled water.
Nucleotides: Cytidine monophosphate (CMP; Sigma) was dissolved in distilled water.

Reagents were filtered through a sterile 0.2µm filter (Nalgene) and were stored at 4°C until use.

5.2.2.2.5 Preparation of salmon sperm DNA

Single-stranded salmon sperm DNA (0.1mg/ml; Sigma) was defrosted immediately prior to addition to culture media and stored at –20°C. Single-stranded DNA is a by-product of late-stage erythrocyte maturation (409) and was added in an attempt to provide a possible substrate for direct nucleotide-importation, as would be possible *in vivo* from within the host's bloodstream and splenic pulp (409).

5.2.2.2.6 Preparation of splenocyte suspensions

The spleen was extracted aseptically from a 9-week-old mouse (killed under terminal anaesthesia as described in 2.2.3.7) and placed in a sterile tissue culture dish, containing 2ml StemPro™-34 medium. The pulp was removed by applying blunt pressure to the capsule and the capsule

subsequently removed from the dish. The suspension was passed through a series of needles in decreasing diameters (18G, 20G, 23G; Terumo) and the suspension centrifuged at 1000 x g for 5 minutes and the supernatant removed. The pellet was resuspended in 150µl StemPro™-34 medium. Splenocyte suspensions were prepared immediately prior to use and were not stored.

5.2.2.2.7 Collection of uninfected mouse blood and preparation of washed eryrthrocyte suspensions

Fresh mouse blood was collected by cardiac puncture under terminal anaesthesia as described above in 5.2.2.1.4.2 other than the mice were not inoculated with *M. haemomuris* prior to exsanguination. Blood was transported to the laboratory under isothermal conditions and within five minutes of collection. When washed erythrocyte suspensions were used, li-heparinised whole blood was diluted with 5ml of StemPro™-34 and centrifuged at 1000 x g for 5 minutes. This washing by centrifugation, removing the supernatant and resuspending the pellet in fresh medium was repeated twice before resuspension of the blood cells, to the volume originally collected, using StemPro™-34 to replace plasma. Erythrocyte suspensions were prepared immediately prior to use and not stored.

5.2.2.2.8 Preparation of murine erythroblast progenitor cell (stem cell) suspensions

A murine cell line of p53-/- erythroblast progenitors was kindly donated by Dr. Ash Toye and Dr. Tim Satchwell, School of Biochemistry, University of Bristol and grown as described by von Lindern, Deiner (406). In brief, cryopreserved cells were seeded at 5E+05 cells/ml into StemPro™-34 medium and supplemented with the following additives:

Expansion protocol:

To expand erythroblast progenitor stem cells for mass cultures, the cells were supplemented with murine recombinant stem cell factor (SCF, R&D Systems, MN, USA, final concentration 100ng/ml), human recombinant erythropoietin (Erypo Cilag AG, Switzerland; final concentration 2U/ml) and dexamethasone (Sigma; final concentration 1µM).

Differentiation protocol:

To differentiate erythroblast progenitor stem cells to erythroblasts, cells were supplemented with human recombinant erythropoietin (Erypo Cilag; final concentration 10U/ml), human recombinant insulin (Sigma; final concentration 10µg/ml) and porcine holotransferrin (kindly provided by Dr. Ash Toye; final concentration 0.5mg/ml).

To avoid potential interference with the subsequent *M. haemomuris* cultivation and avoid additional experimental variables, no antibiotics were used at any time. Mass cultures of erythroblast progenitor cells were obtained by incubation of cells in Costar® flat bottom plates 3516 (Thermo Fisher, recommended working volume of 2000µl per well) in a HERA cell CO_2 incubator (Kendro Laboratory Products, NC, USA) set to 37°C and a CO_2 concentration of 5% (v/v) in room air. Cells were monitored daily using a trypan blue exclusion stain (Gibco) and a haemocytometer. In brief, viable cells were counted as per manufacturer's instructions. Cell suspensions were then spun at 1000 x g for 5 minutes and the pellet resuspended in fresh medium including appropriate additives (expansion protocol) to maintain murine erythroblast progenitors at 1.0-2.0E+06 cells/ml (typically every 24 to 48 hours).

5.2.2.3 Inoculation of in vitro cultures

Prior to inoculation of *in vitro* cultures, culture media and all additives were aliquoted into Costar® flat bottom plates 3536 (Thermo Fisher, recommended working volume 500µl, per well) and plates warmed to 37°C by placing them in the CO_2 incubator (Kendro) for 30 minutes. Unless otherwise specified, 500µl of culture medium was used per well. Wells were inoculated individually with

freshly collected *M. haemomuris*-infected blood, collected from amplifier mice (5.2.2.1.4.2) for each study. Prior to inoculation, the vial was inverted five times and unless otherwise specified, 50µl of *M. haemomuris*-infected mouse blood was added to 450µl of liquid culture media in a laminar flow hood. The method of mixing *M. haemomuris*-infected blood and culture media was varied between studies and is described later.

5.2.2.4 Incubation conditions

Unless otherwise specified, Costar® flat bottom plates 3536 (Thermo Fisher, recommended working volume 500µl) were used to incubate *M. haemomuris* cultures and handled aseptically under laminar air flow. Culture plates were incubated in a HERA cell CO_2 incubator (Kendro) set to 37°C and a CO_2 concentration of 5% (v/v) in room air. The unit was run through the built-in sterilisation protocol prior to starting the experiments. Following inoculation, cultures containing *M. haemomuris* were subject to daily partial (typically 80%) media replacements by carefully removing the supernatant without disturbing the sedimented cells and resuspending the cells to 500µl with fresh media containing the appropriate additives at 37°C.

5.2.2.5 Sampling protocol of in vitro cultures to determine M. haemomuris genome copies per µl culture

Unless otherwise specified, culture wells were sampled after resuspension/media replacement to a 500µl total volume, typically once daily or every other day. Ten microlitres were taken after resuspension and placed into a 1.5ml tube containing 90µl of sterile PBS. Samples were then stored at - 80°C until DNA extraction.

5.2.2.5.1 DNA extraction from culture samples

Total nucleic acid was extracted using an automated, magnetic bead system (Chemagic; Chemagen) described in 4.2.2.3.2.

5.2.2.5.2 QPCR conditions

Conditions of the *M. haemomuris*-specific qPCR were as described in 3.2.2.3.2.

5.2.2.5.3 Assessment of culture media for qPCR inhibitors

To ensure culture media did not inhibit the *M. haemomuris*-specific qPCR assay, a 1:10 dilution of li-heparinised, *M. haemomuris*-infected mouse blood (sourced from an amplifier mouse during the cytokine expression study described in Chapter 4; 4.2.1.6.2.3) in StemPro™-34 was prepared and subject three times to a 1:10 dilution series in either PBS or StemPro-34™. Samples were subject to DNA extraction (see 4.2.2.3.2) and *M. haemomuris*-specific qPCR (3.2.2.3.2) in triplicate and Ct values compared.

5.2.2.5.4 Calculation of *M. haemomuris* genome copies per µl culture media

Relative *M. haemomuris* genome copies per µl culture medium were calculated as described in 2.2.1.2.2.2.2. No normalisation for host control DNA (GAPDH) was performed to account for variable numbers of nucleated cells within the culture experiments over time. Threshold cycles of the GAPDH assay (as part of the duplex assay described in 3.2.2.3.2) were recorded to monitor possible extraction failures or failed reactions. All reactions were run in triplicate and genome copy numbers averaged before data analysis, unless otherwise specified.

5.2.2.6 Study 1 using plateau-phase infected blood and murine erythroblast progenitor cell lines

Six different media compositions were prepared and 450µl aliquots placed into culture wells in duplicate (**Table 1** and Appendix D, **Figure A-D1**).

Media composition ID (duplicate culture well IDs)	Stem cell supplements	Hypoxanthine	Added electrolytes	Stem cells (murine erythroblast progenitors)	*M. haemomuris* dose in 50µl of inoculum per 500µl well*
A1 (A1, A1')	SCF (100ng/ml) EPO (2U/ml) Dexamethasone (1µM)	None	None	1.0E+06 cells/ml	1.87E+05
D1 (D1, D1')	SCF (100ng/ml) EPO (2U/ml) Dexamethasone (1µM)	200µM	None	1.0E+06 cells/ml	1.87E+05
D2 (D2, D2')	SCF (100ng/ml) EPO (2U/ml) Dexamethasone (1µM)	100µM	None	1.0E+06 cells/ml	1.87E+05
D3 (D3, D3')	SCF (100ng/ml) EPO (2U/ml) Dexamethasone (1µM)	50µM	None	1.0E+06 cells/ml	1.87E+05
E1 (E1, E1')	SCF (100ng/ml) EPO (2U/ml) Dexamethasone (1µM)	None	25µg/ml $MgCl_2$ 41µg/ml $CaCl_2$	1.0E+06 cells/ml	1.87E+05
E2 (E2, E2')	SCF (100ng/ml) EPO (2U/ml) Dexamethasone (1µM)	100µM	25 µg/ml $MgCl_2$ 41µg/ml $CaCl_2$	1.0E+06 cells/ml	1.87E+05
A2 (A2)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	1.0E+06 cells/ml	5.98E+05 (* here per 2000µl well)

Table 46 *Composition of culture media used in the in vitro cultivation study 1, using M. haemomuris-infected blood to try to infect murine erythroblast progenitor cell lines. M. haemomuris was collected during plateau-phase bacteraemia from an M. haemomuris-infected C57BL/6 mouse at 31 dpi and 50µl blood added to 450µl of media in duplicate, except for medium A2, that was trialled at a final volume of 2000µl. SCF, stem cell factor; EPO, human recombinant erythropoietin.*

Li-heparin-anticoagulated blood was collected at 31 dpi from a 15-week-old, female, *M. haemomuris*-infected C57BL/6 mouse and contained 3.73E+03 haemoplasma genome copies per µl as determined by qPCR. Duplicate culture wells containing media A1, B1, B2, D1, D2, D3, E1 and E2 (defined in **Table 46**) were inoculated with 50µl of *M. haemomuris*-infected blood each, followed by gentle pipetting to mix blood and culture media. Wells were subject to partial media replacements daily, followed by gentle pipetting to resuspend sedimented cells in culture media. Wells were sampled at 0, 2, 4, 6 and 8 dpi, immediately following passaging (discarding half of the well volume, followed by 1:1 dilution in fresh media) of the growing erythroblast progenitors for monitoring of *M. haemomuris* genome copies per µl culture media. Passaging into new medium was required to ensure erythroblast suspensions remained between 1.0E+06 and 2.0E+06 cells/ml as required for optimal viability and growth (406). When calculating total *M. haemomuris* genome copies per µl medium, passaging was accounted for by multiplying genome copies with the dilution factor. Total culture time was eight days.

An additional, single, larger well 'A2' (Costar® flat bottom plates 3516, ThermoFisher, **Table 46**) was included and inoculated with 160µl of infected blood to pilot the differentiation protocol of the murine erythroblast progenitors that leads to them being differentiated enough to produce haemoglobin (5.2.2.2.8) and become erythroblasts. Inoculation was followed by gentle pipetting to mix blood and culture media. Well A2 was subject to partial media replacement daily. Media replacement was followed by gentle pipetting to resuspend sedimented cells in culture media. Cells

in well A2 were passaged (discarding half of the well volume, followed by 1:1 dilution in fresh media) at 2, 4, 6, 8 and 10 dpi and sampled at 0, 1, 2, 4, 6, 8, 10, 12, 14, 17, 18 and 20 dpi. When calculating total *M. haemomuris* genome copies per µl medium, passaging was accounted for by multiplying genome copies with the dilution factor. Total culture time was twenty days.

5.2.2.6.1 Sentinel mice for study 1

At 8 dpi of culture wells, well 'A2' was split into 2 aliquots and centrifuged at 1000 x g for 5 minutes. One aliquot was resuspended in 2 x 200 µl of fresh StemPro™-34 medium, forming two inocula. The two inocula were inoculated intraperitoneally into two sentinel mice (one inoculum dose per mouse) as outlined in **Table 47**. Inoculation procedure was as described above in 5.2.2.1.5.1 and monitoring as described in 5.2.2.1.5.2. The second aliquot was resuspended in 2ml of culture medium A2 and maintained as described above in 5.2.2.6.

Sentinel mouse ID	Inoculum composition (culture well in **Table 1**)	Dpi of culture at the time of sentinel mouse inoculation	*M. haemomuris* dose in 200µl of inoculum per mouse
S1, S2	A2	8	1.50E+05

Table 47 *Origin, composition and days post inoculation (dpi) of inocula used to infect sentinel mice during in vitro cultivation study 1.*

5.2.2.7 Study 2 using pre-peak infected blood, splenocyte suspensions, differentiated erythroblasts, whole blood and erythrocyte suspensions

Eight different media compositions were prepared and 450µl aliquots placed into culture wells in duplicate (**Table 48** and Appendix D, **Figure A-D2**).

Media composition ID (duplicate culture well IDs)	Stem cell supplements	Hypoxanthine	Blood components	Stem cells (differentiated murine erythroblasts)	Splenocytes	*M. haemomuris* dose in 50µl of inoculum per 500µl well
F (F, F')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	2.0E+06 per ml	50µl primary splenocyte suspension (no cell count)	3.29E+07
G (G, G')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	Washed erythrocytes (equivalent to 50µl blood)	2.0E+06 per ml	None	3.29E+07
H (H, H')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	50µl whole mouse blood (uninfected)	2.0E+06 per ml	None	3.29E+07
I (I, I')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	Washed, uninfected, mouse erythrocytes (equivalent to 50µl blood)	None	None	3.29E+07
J (J, J')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	50µl whole mouse blood (uninfected)	None	None	3.29E+07
K (K, K')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	None	None	3.29E+07
L (L, L')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 1mg/ml	100µM	None	2.0E+06 per ml	None	3.29E+07
X (X, X')	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	200µl whole mouse blood (uninfected)	None	None	9.86E+07 (in 150µl inoculum)

Table 48 *Composition of culture media used in the in vitro cultivation study 2, using M. haemomuris-infected blood to try to infect differentiated murine erythroblast cell lines, naïve murine blood, washed murine erythrocytes and splenocyte suspensions. M. haemomuris was collected during exponential M. haemomuris growth prior to peak-phase bacteraemia from an M. haemomuris-infected C57BL/6 mouse at 6 days post inoculation and 50µl blood added to 450µl of media in duplicate, except for medium X, that comprised 200µl of uninfected whole mouse blood and 150 µl medium and had 150 µl M. haemomuris-infected blood added to it. EPO, human recombinant erythropoietin.*

Li-heparin-anticoagulated blood was collected at 6 dpi from a 12-week-old, female, *M. haemomuris*-infected C57BL/6 mouse and contained 6.57E+05 *M. haemomuris* genome copies per µl blood as determined by qPCR. Blood collected during exponential *in vivo* growth prior to peak parasitaemia ('pre-peak', data not shown) was chosen over plateau-phase collected blood (see **Figure 20** ff. for phases of *M. haemomuris* infection). This was because it was not possible to differentiate viable haemoplasmas from non-viable haemoplasma DNA when calculating haemoplasma doses by PCR, and thus there was a possibility of potentially overestimating inoculum doses for *in vitro* cultivation. By using organisms collected during the exponential phase of growth, it was hoped that these most likely represented viable haemoplasmas. Duplicate culture wells containing media F, G, H, I, J and K were inoculated with 50µl of *M. haemomuris*-infected blood each, followed by centrifugation at

1000 x g for 5 minutes to enhance cell-to-cell contact and resuspension by gentle pipetting to mix blood and culture media. Wells were subject to partial media replacements daily, followed by gentle pipetting to resuspend sedimented cells in culture media. Wells were sampled at 0, 1, 2 and 3 dpi, without passaging. Passaging, which effectively resulted in dilution to maintain steady concentrations of the stem cells, was omitted due to concerns over providing insufficient cell densities for growing haemoplasmas. Total culture time was reduced to three days, compared to the 8 days in *in vitro* cultivation study 1, due to concerns arising from haemolysis (and a drop in *M. haemomuris* genome copies per µl medium) that was occurring in the wells when they contained more erythrocytes than included in the inoculum dose of 50µl.

An additional medium composition 'X' was included and inoculated with 150µl *M. haemomuris*-infected blood to pilot the effect of higher erythrocyte densities (medium composition see **Table 48**) on haemoplasma growth. Inoculation and the protocol of sampling of duplicate wells containing medium 'X' were identical wells containing media 'F' to 'K' as described above.

5.2.2.7.1 Sentinel mice for study 2

At 12 hours post inoculation of culture wells, well 'X'' was centrifuged at 1000 x g for 5 minutes and resuspended in 200µl of StemPro™-34 medium, forming one inoculum. This inoculum was inoculated intraperitoneally into one sentinel mouse as outlined in **Table 49**. At 24 hours post inoculation of culture wells, well 'X' underwent identical processing and inoculation into another sentinel mouse.

At 2 dpi of culture wells, duplicate wells (indicated with an apostrophe) were pooled in the following order: G' + I' + L', H' + J' whilst K' and F' remained separate. Pooled and separate well contents were centrifuged at 1000 x g for 5 minutes and resuspended in 200µl of StemPro™-34 medium, forming four inocula. These four inocula were inoculated intraperitoneally into four sentinel mice (one inoculum per mouse) as outlined in **Table 49**. The inoculation procedure was as described above in 5.2.2.1.5.1 and monitoring was as described in 5.2.2.1.5.2.

Sentinel mouse ID	Inoculum composition (culture well)	Dpi of culture at the time of sentinel mouse inoculation	*M. haemomuris* dose in 200µl of inoculum per mouse
S3	X'	0.5 (12 hours)	1.98E+06
S4	X	1 (24 hours)	not detected
S5	G' + I' + L'	2	8.42E+07
S6	H' + J'	2	6.07E+07
S7	K'	2	7.92E+07
S8	F'	2	5.67E+07

Table 49 *Origin, composition and days post inoculation (dpi) of inocula used to infect sentinel mice during in vitro cultivation study 2.*

5.2.2.8 Study 3 (viability study) using StemPro™-34 without cellular supplementation

As no haemoplasma growth had been observed in the experiments thus far, it was decided to determine *M. haemomuris* viability in a medium composition identical to that of K (containing no cellular components, medium composition see **Table 48**) over time to determine if the medium was likely to support and maintain *M. haemomuris* organisms even in the absence of growth for longer than two days as it had been assessed in study 2. No cellular components were added as no increased viability had been observed in cell-supplemented media. Five identical wells were prepared as shown in **Table 50**.

Media composition ID (replicate culture well IDs)	Stem cell supplements	Hypoxanthine	Blood components	Stem cells	Splenocytes	*M. haemomuris* dose in 50µl of inoculum per 500µl well
V (V2, V4, V6, V8, V10)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	None	None	8.73E+06

Table 50 *Composition of culture media used in the in vitro cultivation study 3 (viability study), using M. haemomuris-infected blood in StemPro™-34 without cellular additives. M. haemomuris was collected prior to peak-phase bacteraemia from an M. haemomuris-infected C57BL/6 mouse at 3 dpi and 50µl blood added to 450µl of medium in five identical wells. EPO, human recombinant erythropoietin.*

Li-heparin-anticoagulated blood was collected at 3 dpi from a 9-week-old, female, *M. haemomuris*-infected C57BL/6 mouse and contained 1.75E+05 *M. haemomuris* genome copies per µl blood as determined by qPCR. Five identical wells were inoculated with 50µl of *M. haemomuris*-infected blood each, followed by gentle rocking of the plate to mix blood and culture media. The mixing technique was changed compared to previous studies as concerns existed as to whether the mixing was resulting in, or contributing to, the haemolysis. To eliminate any potential effect that removing and replacing 10µl of culture every day could have on inoculum viability (due to having to resuspend cells to create a uniform suspension), no daily sampling was performed. Wells were subject to partial media replacements daily, followed by gentle rocking of the plate to resuspend blood and haemoplasmas in the culture media.

5.2.2.8.1 Sentinel mice for study 3

At 2, 4, 6, 8 and 10 dpi of culture wells, contents of one well were removed, centrifuged at 1000 x g for 5 minutes and resuspended in 200µl of the same medium (V) to form an inoculum. Each inoculum generated on days 2, 4, 6, 8 and 10 was inoculated intraperitoneally into one sentinel mouse as outlined in **Table 51**. Inoculation procedure was as described above in 5.2.2.1.5.1 and monitoring as described in 5.2.2.1.5.2.

Sentinel mouse ID	Inoculum composition (culture well)	Dpi of culture at the time of sentinel mouse inoculation	*M. haemomuris* dose in 200µl of inoculum per mouse
S9	V2	2	n/d
S10	V4	4	n/d
S11	V6	6	n/d
S12	V8	8	n/d
S13	V10	10	n/d

Table 51 *Origin, composition and days post inoculation (dpi) of inocula used to infect sentinel mice during in vitro cultivation study 3.*

5.2.2.9 Study 4 (RNA study) using StemPro™-34 without cellular supplementation

A decision was made to monitor and quantify *M. haemomuris* RNA during the *in vitro* cultivation attempts, as it was hoped that RNA might potentially better reflect *M. haemomuris* viability. This is because bacterial RNA transcripts are thought to be short-lived and hence represent viable organisms, as they would not be produced by dead bacterial cells (410), although their usefulness for detecting viable pathogens has recently has been called into question (411). However, an RNA assay was hoped to lead to a reduced requirement for sentinel mice for assessment of haemoplasma viability. An experiment was designed to repeat the culture conditions outlined in 5.2.2.8 and quantify RNA as well as inoculating wells into sentinel mice (at 3 and 7 dpi) in order to see if a fall in RNA levels occurred that could be consistent with a reduction in the viable haemoplasmas under *in vitro* conditions.

5.2.2.9.1 Digestion of DNA and adaptation of the qPCR assay for RNA work (RT-qPCR)

The *in vivo* infection kinetics of *M. haemomuris* RNA and DNA were determined in a pilot experiment, using blood samples (2 x 10µl) collected daily from 0 to 14 dpi with *M. haemomuris* in one 13-week-old mouse. Blood samples, diluted in 90µl of PBS each, were stored at -80°C until TNA extraction. The *M. haemomuris*-specific duplex qPCR assay (3.2.2.3.2) had been designed to amplify the DNA of the 16S rRNA gene of *M. haemomuris* and a transcribed region of the cat/mouse GAPDH gene, hence no amendment of primer sequences was required to adapt the assay to detect and amplify cDNA transcripts from the same genes following reverse transcriptase (RT) reactions.

One sample of 10µl blood underwent TNA extraction as described before in 5.2.2.5.1 and was stored at –80°C after extraction and before qPCR. The other 10µl blood sample underwent identical TNA extraction, followed by DNA-digestion with DNase (1U/µl, RQ1 RNase-free; Thermo Fisher) as per manufacturer's instructions. In brief, 30µl of TNA, 5µl of RNA-se free water, 4µl of 10X RQ1 reaction buffer and 1µl of DNAse were gently mixed and incubated at 37°C for 30 minutes. One microlitre of RQ1 stop solution (EDTA) was added and the mixture incubated at 65°C for 10 minutes to inactivate the DNAse in the sample now containing the total RNA in a volume of 40µl. Reverse transcription was performed using a MJ Mini Gradient Termal Cycler and ImProm II Reverse Transcriptase (Promega) to the manufacturer's instructions. In brief, 5µl of total RNA were combined with ImProm II 5X reaction buffer, 3mM $MgCl_2$, dNTPs (0.5mM each), random hexamers (25ng/µl) and ImProm II reverse transcriptase to a total volume of 20 µl. The mixture was subject to the following thermal cycling protocol: 20°C for 5 minutes, 42°C for 30 minutes and 70°C for 15 minutes. The resulting 20µl of cDNA was added to 30µl of RNase-free water and stored at −80°C. Five microlites of the original total RNA sample were also diluted in 50µl RNAse-free water and stored at –80°C until qPCR.

Both sets of nucleic acid samples, generated from the originally collected 2 x 10µl blood samples, now either containing cDNA and DNA-free RNA or directly extracted TNA (extracted separately, diluted 3:4 and 1:10 to account for DNA-digestion and reverse transcription) were subject to *M. haemomuris*-specific duplex qPCR (3.2.2.3.2) to assess the DNA and RNA transcripts over the time course of *M. haemomuris* infection and the efficiency of DNA-digestion. Positive and negative (water) controls were included. Calculations of *M. haemomuris* genome copies per µl blood and 16S RNA copies per µl blood were performed as described before in 5.2.2.5.4.

5.2.2.9.2 *In vitro* cultivation study 4

Two identical wells were prepared with culture medium as shown in **Table 52**.

Media composition ID (duplicate culture well IDs)	Stem cell supplements	Hypoxanthine	Blood components	Stem cells	Splenocytes	*M. haemomuris* dose in 50µl of inoculum per 500µl well
W (W1, W2)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	None	None	3.92E+08 (equivalent to 1.99E+09 16S RNA copies as determined by RT-qPCR in 5.2.2.9.1)

Table 52 *Composition of culture media used in the in vitro cultivation study 4 (RNA study), using M. haemomuris-infected blood in StemPro™-34 without cellular additives. M. haemomuris was collected during exponential growth prior to peak-phase bacteraemia from an M. haemomuris-infected C57BL/6 mouse at 7 dpi and 50 µl blood added to 450 µl of medium in two identical wells. EPO, human recombinant erythropoietin.*

Li-heparin-anticoagulated blood was collected at 7 dpi from a 14-week-old, female, *M. haemomuris*-infected C57BL/6 mouse and contained 7.84E+06 *M. haemomuris* genome copies per µl blood as determined by qPCR. Duplicate blood samples collected from the same mouse undergoing RT-qPCR

as described above in 5.2.3.12.1 were determined to contain 3.99E+07 copies of *M. haemomuris* 16S RNA at the same time. Two identical wells were inoculated with 50µl of *M. haemomuris*-infected blood each, followed by gentle rocking of the plate to mix blood and culture media. Wells were subject to partial (300µl) media replacements daily, followed by gentle rocking of the plates to resuspend sedimented cells in culture media. Suspended cells were then sampled at 0, 1, 2, 3, 4, 5, 6 and 7 dpi. Total culture time was three days for the first well 'W1' and seven days for the second well, 'W2'.

5.2.2.9.3 Sentinel mice for study 4

At 3 and 7 dpi of the culture wells, the contents of one well were removed, centrifuged at 1000 x g for 5 minutes and resuspended in 200µl of the same medium ('W', see **Table 52**) to form an inoculum. Each inoculum generated at 3 and 7 dpi was inoculated intraperitoneally into one sentinel mouse as outlined in **Table 53**. The inoculation procedure was as described above in 5.2.2.1.5.1 and monitoring as described in 5.2.2.1.5.2.

Sentinel mouse ID	Inoculum composition (culture well)	Dpi of culture at the time of sentinel mouse inoculation	*M. haemomuris* dose in 200µl of inoculum per mouse
S14	W1	3	8.15E+08
S15	W2	7	6.64E+08

Table 53 *Origin, composition and days post inoculation (dpi) of inocula used to infect sentinel mice during in vitro cultivation study 4.*

5.2.2.10 Study 5 (purine/pyrimidine study) using nucleobases, nucleotides, single-stranded DNA and whole blood supplementation

To determine if *M. haemomuris* exhausted supplies of replaceable substrates or was inhibited somehow by its own growth, experiments were designed to assess whether growth in previous media compositions could be improved by the addition of nucleobases, nucleosides, nucleotides, single-stranded DNA or by dilution and the addition of naïve mouse blood at 3 dpi of *in vitro* cultures. Eight wells were prepared, with duplicate wells for each medium composition, as shown in **Table 54** and Appendix D, **Figure A-D3**.

Media composition ID (duplicate culture well IDs)	Stem cell supplements	Hypoxanthine	Blood components	Nucleobases, nucleosides and nucleotides	SsDNA (salmon sperm)	*M. haemomuris* dose in 50µl of inoculum per 500µl well
N (N1, N2)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	None	None	1.29E+09
NUCLEO (NUC1, NUC2)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	Adenine (100mg/l), Guanine (100mg/l), Thymine (100mg/l), Uracil (100mg/l), Cytosine (100mg/l), Cytidine (100mg/l), CMP (100mg/l)	None	1.29E+09
SALMON (SAL1, SAL2)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	None	None	100mg/l	1.29E+09
BLOOD (BLO1, BLO2; BLO2 then split into BLO2a and BLO2b at 3dpi)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	50µl whole mouse blood (uninfected) at 3 dpi, coinciding with 1:1 dilution	None	None	1.29E+09

Table 54 *Composition of culture media used in the in vitro cultivation study 5 (purine/pyrimidine study), using M. haemomuris-infected blood in StemPro™-34 with additional nucleobases, nucleosides, single-stranded DNA (ssDNA) and fresh blood replacement during media expansions. M. haemomuris was collected prior to peak-phase bacteraemia from an M. haemomuris-infected C57BL/6 mouse at 7 dpi and 50µl blood added to 450µl of medium in two identical wells. EPO, human recombinant erythropoietin; CMP, cytosine monophosphate; ssDNA, single-stranded DNA.*

Li-heparin-anticoagulated blood was collected at 7 dpi from a 12-week-old, female, *M. haemomuris*-infected C57BL/6 mouse and contained 2.57E+07 *M. haemomuris* genome copies per µl blood as determined by qPCR. Two identical wells were inoculated with 50µl of *M. haemomuris*-infected blood each, followed by gentle rocking of the plate to mix blood and culture media. Wells were subject to partial (300µl) media replacements daily, followed by gentle rocking of the plates to resuspend sedimented cells in culture media. Suspended cells were then sampled at 0, 1, 2, 3, 4, 5, 6, 7 and 8 dpi. Total culture time was three days for the first well of each medium (indicated by '1' in the well name) and eight days for the second well (indicated by '2' in the well name). At 3 dpi of culture wells, one well of each medium composition ('1') was inoculated intraperitoneally into one sentinel mouse as outlined in 5.2.2.1.5.1. The second well of each composition ('2') was maintained as before except for well 'BLO2'. At 3 dpi of culture wells, well 'BLO2' was split in two aliquots of 250 µl and resuspended in 200µl of the same medium with additional 50µl of uninfected mouse blood, sourced from a naïve mouse as described above in 5.2.2.2.7. This resulted in two culture wells, 'BLO2a' and 'BLO2b' that were maintained and sampled as the remaining wells as illustrated in **Figure 41**.

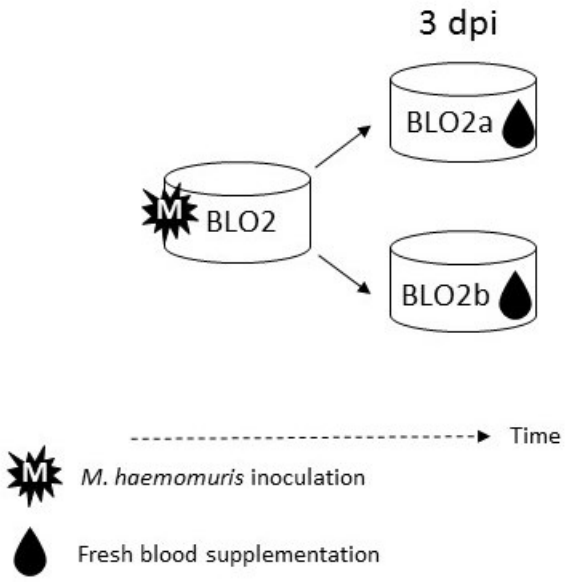


Figure 41 *Schematic of the media expansion and blood replacement step at 3 days post culture inoculation of the in vitro cultivation study 5. Blood, infected with M. haemomuris was sourced from a donor mouse and used to inoculate the original well, undergoing partial media replacement and daily sampling. At 3 dpi, one well containing fresh blood was split equally into two new wells, each of which contained 50µl of fresh (uninfected) blood. Dpi, days post inoculation (of culture wells).*

5.2.2.10.1 Sentinel mice for study 5

At 3 dpi of the culture wells, the contents of one duplicate well per medium composition were removed, centrifuged at 1000 x g for 5 minutes and resuspended in 200µl of the same medium (inoculum compositions N, NUCLEO, SALMON, BLOOD; see **Table 54** for composition descriptions) to form an inoculum. Each inoculum generated was inoculated intraperitoneally into one sentinel mouse as outlined in **Table 55**. The inoculation procedure was as described above in 5.2.2.1.5.1 and monitoring as described in 5.2.2.1.5.2.

At 8 dpi of culture wells, well 'BLO2a' was inoculated intraperitoneally into one sentinel mouse (**Table 55**) using identical inoculation generation procedure, inoculation procedure and monitoring of the sentinel mouse. The remainder of the wells were discarded after sampling and the experiment terminated at 8 dpi.

Sentinel mouse ID	Inoculum composition (culture well)	Dpi of culture at the time of sentinel mouse inoculation	*M. haemomuris* dose in 200µl of inoculum per mouse
S16	N1	3	3.64E+09
S17	NUC1	3	6.59E+08
S18	SAL1	3	8.49E+09
S19	BLO1	3	4.91E+09
S20	BLO2a	8	1.45E+09

Table 55 *Origin, composition and days post inoculation (dpi) of inocula used to infect sentinel mice during in vitro cultivation study 5.*

5.2.2.11 Study 6 (continuous culture pilot) using serial media expansions and blood replacement

Based on the results of passaging *M. haemomuris* cultures into media containing fresh mouse blood, it was decided to repeat the experiment comprising of media expansion combined with addition of fresh mouse blood at 2 dpi of culture wells. Three identical wells were prepared as shown in **Table 56**.

Media composition ID (duplicate culture well IDs)	Stem cell supplements	Hypoxanthine	Blood components	*M. haemomuris* dose in 50µl of inoculum per 500µl well
CONTIN (CON1, CON2, CON3; all to infect new recipient wells at 2 dpi)	EPO 10U/ml Insulin 10µg/ml Holotransferrin 0.5mg/ml	100µM	50µl whole mouse blood (uninfected) at 2 dpi, coinciding with 1:10 dilution	2.84E+08

Table 56 *Composition of culture media used in the in vitro cultivation study 6 (continuous pilot), using M. haemomuris-infected blood in StemPro™-34 with fresh blood replacement during media expansions. M. haemomuris was collected prior to peak-phase bacteraemia from an M. haemomuris-infected C57BL/6 mouse at 7 dpi and 50µl blood added to 450µl of medium in two identical wells. EPO, human recombinant erythropoietin.*

Li-heparin-anticoagulated blood was collected at 7 dpi from a 10-week-old, female, *M. haemomuris*-infected C57BL/6 mouse and contained 5.69E+06 *M. haemomuris* genome copies per µl blood as determined by qPCR. Three identical wells were inoculated with 50µl of *M. haemomuris*-infected blood each, followed by gentle rocking of the plate to mix blood and culture media. Wells were subject to partial (300µl) media replacements daily, followed by gentle rocking of the plates to resuspend sedimented cells in culture media. Suspended cells were then sampled at 0, 1, 2, 3 and 4 dpi. Total culture time was four days. At 2 dpi of the culture wells, 50µl of each infected culture was transferred into a new recipient well, containing 50µl of fresh (uninfected) blood and diluted with medium to the original volume of 500µl. Uninfected blood was sourced from a naïve mouse, as described above in 5.2.2.2.7. This media expansion and blood replacement step resulted in two new culture wells (recipient and donor well) for each original well. The recipient well underwent identical partial media replacement and sampling as before for another two days. The donor well (original culture) was reconstituted to the original volume of 500µl and maintained for another day. A schematic of this part of the experiment is given in **Figure 42**. At 4 dpi of the culture wells, one culture well (recipient well from CON1) was inoculated intraperitoneally into one sentinel mouse.

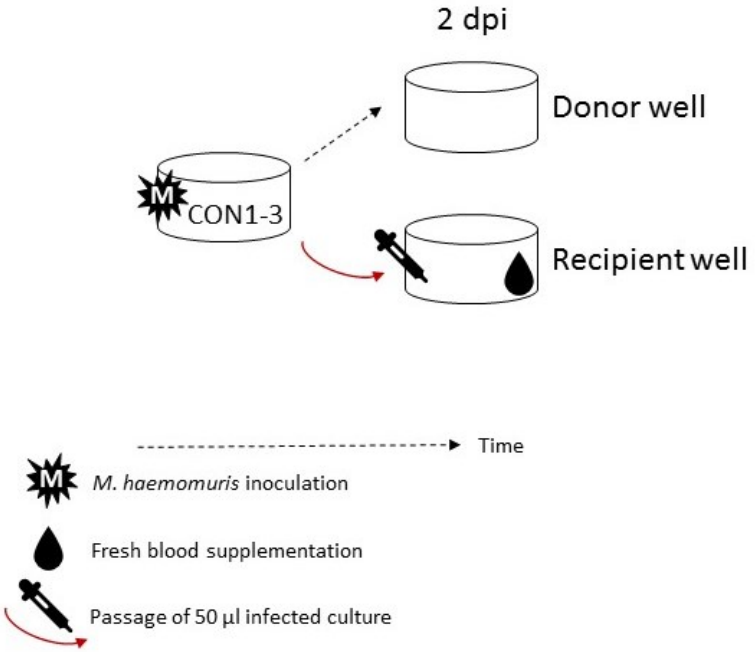


Figure 42 *Schematic of the media expansion and blood replacement step at 2 days post culture inoculation of the in vitro cultivation study 6 (continuous culture pilot). Blood, infected with M. haemomuris was sourced from a donor mouse and used to inoculate the original well. At 2 dpi, 50µl of infected culture was transferred into a new well, containing 50µl of fresh (uninfected) blood and diluted with medium to a volume of 500µl. Dpi, days post inoculation (of culture wells).*

5.2.2.11.1.1 Sentinel mice

At 4 dpi of the culture wells, the contents of one well were removed, centrifuged at 1000 x g for 5 minutes and resuspended in 200µl of the same medium (inoculum composition CONTIN, see **Table 56**) to form an inoculum that was then inoculated intraperitoneally into one sentinel mouse as outlined in **Table 57**. The inoculation procedure was as described above in 5.2.2.1.5.1 and monitoring as described in 5.2.2.1.5.2.

Sentinel mouse ID	Inoculum composition (culture well)	Dpi of culture at the time of sentinel mouse inoculation	*M. haemomuris* dose in 200µl of inoculum per mouse
S21	CON1 (recipient well)	4	7.66E+09

Table 57 *Origin, composition and days post inoculation (dpi) of inocula used to infect sentinel mice during in vitro cultivation study 6 (continuous culture pilot).*

5.3 RESULTS

5.3.1.1 Intra-assay and inter-assay variation of the M. haemomuris-specific qPCR assay

Intra-assay % CVs for each dilution are presented in **Table 58**.

Dilution #	Mean Ct	Stdev Ct	%CV (Ct)	Mean relative *M. haemomuris* genome copies	Stdev of relative *M. haemomuris* genome copies	%CV (genome copies)	Equivalent variation in relative *M. haemomuris* genome copies
1	22.5	0.060	0.27	1.55E+05	6.70E+03	4.33	6.70E+03
1:10	25.7	0.118	0.46	1.76E+04	1.50E+03	8.50	1.50E+03
1:100	28.9	0.185	0.64	1.88E+03	2.77E+02	14.73	2.77E+02
1:1000	31.8	0.465	1.46	2.67E+02	8.89E+01	33.31	8.89E+01

Table 58 *Intra-assay coefficients of variation (% CV) as based on means and standard deviations (stdev) for each of the four amplicon dilutions. There is a typical increase in % CV with increasing Ct values (or lower genome copy numbers). However, as qPCR follows exponential amplification, even gradually decreasing % CVs are equivalent to increasing standard deviations when calculated on M. haemomuris genome copies rather than Ct values. This means that the assay's ability to quantify M. haemomuris genome copies decreases with increasing template concentrations in absolute numbers.*

As expected, the %CV increased with increasing Ct values, reflecting lower repeatability of Ct values at higher template concentrations. Due to the exponential nature of the qPCR reaction, even gradually decreasing %CVs of Ct values are equivalent to higher standard deviations and hence higher variability of *M. haemomuris* genome copies when expressed in absolute numbers. This means that at the lowest template concentration, the range of measured Ct values was equivalent to 8.89E+01 *M. haemomuris* genome copies, and at the highest template concentration, the range of measured Ct values was equivalent to 6.70E+03 *M. haemomuris* genome copies despite the %CVs being lower at higher template concentrations. The %CVs and equivalent *M. haemomuris* genome copy numbers highlighted important considerations for the interpretation of any *in vitro* culture results relating to *M. haemomuris* genome copy quantification by qPCR, to avoid wrongly interpreting any variation of the qPCR assay as *M. haemomuris* growth.

In the subsequent studies, growth was only said to be identified when differences in *M. haemomuris* genome copies per µl culture medium were clearly above the assay's limit of quantification (greater Ct difference than calculated %CV) for the obtained Ct value of the three qPCR replicates. Where Ct values fell in-between or outside the range of the dilutions used for the current study, linear extrapolation (graph not shown) was used to define cut-off values for *M. haemomuris* growth.

Inter-assay %CVs for each dilution are presented in **Table 59** and showed similar values, however to avoid introducing variability due to sample storage, samples from all cultivation studies were typically extracted and subject to qPCR on the same day, using the same mix of qPCR reagents.

Dilution #	%CV (Ct)	%CV (genome copies)	Equivalent variation in relative *M. haemomuris* genome copies
1	0.26	4.15	6.42E+03
1:10	0.54	10.05	1.77E+03
1:100	0.88	19.12	3.59E+02
1:1000	1.71	38.59	1.03E+02

Table 59 *Inter-assay coefficients of variation (% CV) as based on means and standard deviations for each of the four amplicon dilutions from both experimental days.*

Whole intra-assay %CV calculated on Ct values was 0.70%, on the same day, and whole inter-assay % CV was 0.79%, spanning both days, independently of the dilution used.

5.3.1.2 *Application to the M. haemomuris*-specific qPCR assay to culture media preparations

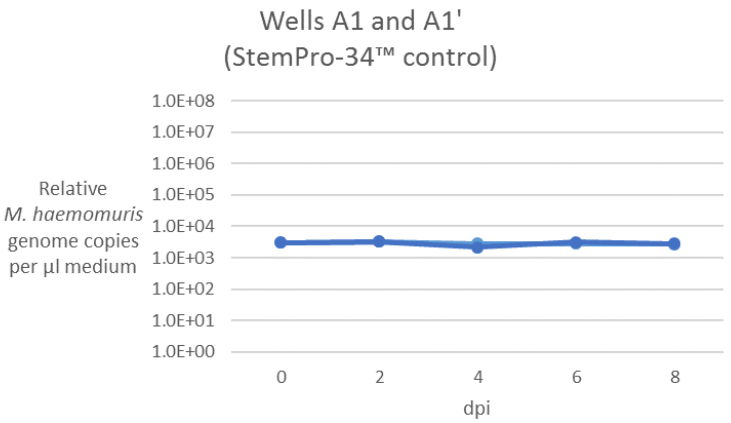
No inhibition of the *M. haemomuris*-specific qPCR assay was observed using mouse blood dilutions of 1:100 to 1:10,000 in StemPro™-34 compared to dilution in PBS following creation of a stock of 'mock culture sample' of 1:10 dilution of li-heparinised mouse blood in StemPro-34™. Threshold cycle values derived to assess qPCR performance in PBS and StemPro™-34 are summarised in **Table 60**.

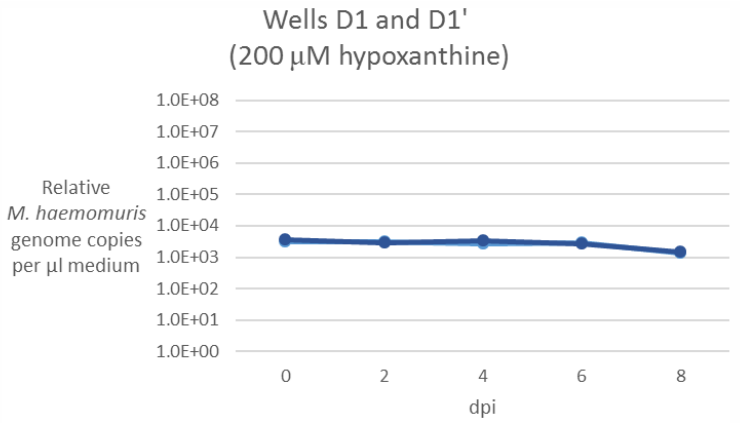
Template	Ct			Δ Ct
1:10 dilution of Stock in PBS	20.09	20.02	20.39	-
1:10 dilution of Stock in StemPro-34™	20.09	20.88	20.28	-
1:100 dilution of Stock in PBS	23.67	23.43	23.27	3.29
1:100 dilution of Stock in StemPro-34™	23.66	23.74	23.61	3.50
1:1000 dilution of Stock in PBS	27.19	27.09	27.73	3.88
1:1000 dilution of Stock in StemPro-34™	27.18	27.21	28.05	3.81

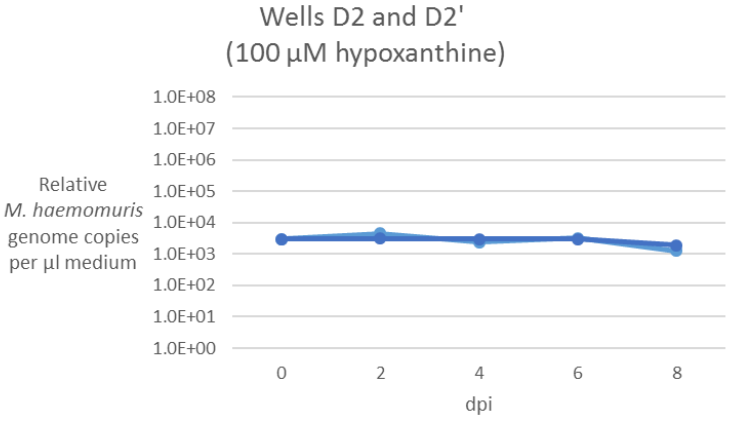
Table 60 *TEX-threshold cycle values (Ct) for the M. haemomuris-specific qPCR assay on a dilution series of a 'mock culture sample' comprising a 1:10 dilution of M. haemomuris-infected mouse blood in StemPro™-34 (Stock) in subsequent 1:10, 1:100 and 1:1000 dilutions of PBS prior to DNA extraction from 100 µl sample through the Chemagic (Chemagen) system as described above in 5.2.2.5.1. The difference in Ct between average Ct values of each dilution with the same diluent is indicated as Δ Ct and consistent with 3.3 Ct being equivalent to a 1:10 dilution.*

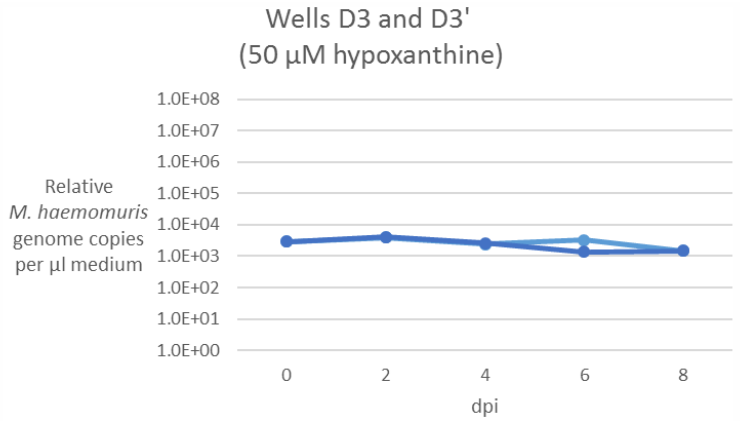
5.3.1.3 Results of in vitro cultivation study 1 using plateau-phase infected blood and murine erythroblast progenitor cell lines

There was no growth detected in any of the 500µl wells inoculated during *in vitro* cultivation study 1 (5.2.2.6) using *M. haemomuris*-infected blood collected from a C57BL/6 mouse at 31 dpi (plateau phase) and combinations of murine erythroblast progenitor stem cells, stem cell supplements, electrolytes and hypoxanthine (**Figure 43**). All mean relative *M. haemomuris* genome copies per µl culture medium at 8 dpi of culture wells were lower when compared to *M. haemomuris* genome copies in the same medium at 0 dpi of culture wells.


Wells A1 and A1'
(StemPro-34™ control)
Relative
M. haemomuris
genome copies
per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
dpi


Wells D1 and D1'
(200 µM hypoxanthine)
Relative
M. haemomuris
genome copies
per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
dpi


Wells D2 and D2'
(100 µM hypoxanthine)
Relative
M. haemomuris
genome copies
per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
dpi


Wells D3 and D3'
(50 µM hypoxanthine)
Relative
M. haemomuris
genome copies
per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
dpi

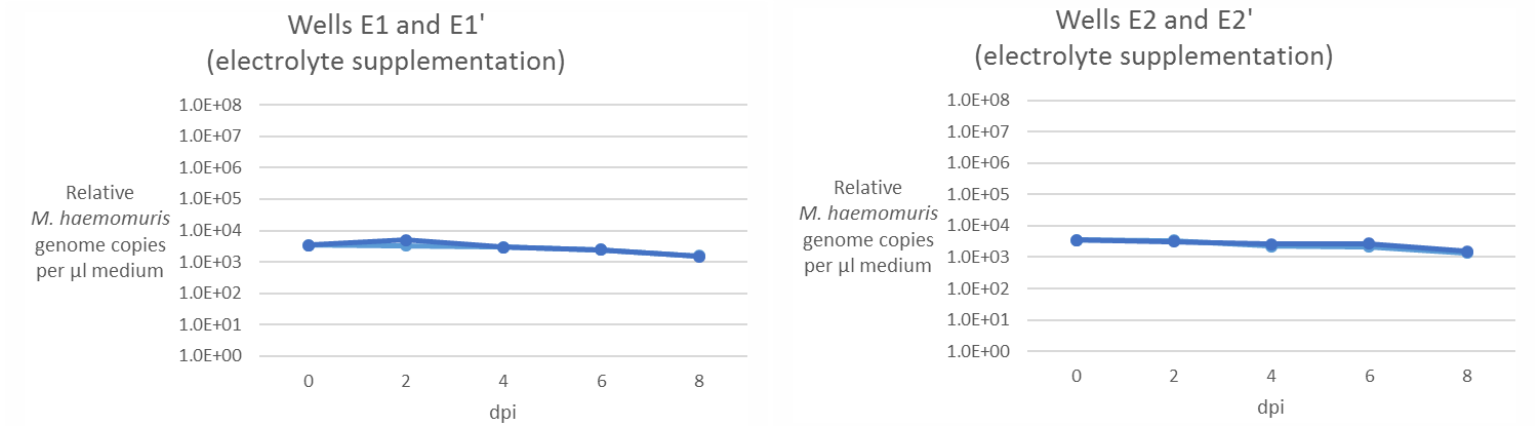


Figure 43 *M. haemomuris genome copies during in vitro cultivation study 1, showing amplification plots of culture wells A1 and A1', D1 and D1', D2 and D2', D3 and D3', E1 and E1' as well as E2 and E2' after inoculation of 50µl M. haemomuris-infected mouse blood into 450µl of prepared media A1, D1, D2, D3, E1 and E2. Media compositions are given in* ***Table 1****.Days post inoculation (dpi; of wells) are plotted on the x-axis and relative haemoplasma genome copies per µl medium are plotted on the y-axis. Connected dots of the same colour represent individual culture wells and where indistinguishable are overlapping each other. Wells were subject to partial media replacements daily and passaged, equivalent to a 1:1 dilution every other day. Dilution factors were accounted for before creating the plots. As zero values cannot be plotted on logarithmic scales, values of one (1.0E+00) M. haemomuris copy per µl blood (and qPCR reaction) are being treated as the equivalent of zero on this and subsequent graphs within this chapter.*

Well A2 contained an increased amount of inoculum (*M. haemomuris* infected blood from the same C57BL/6 mouse as used for the 500 µl wells above) and different stem cell supplements including transferrin (see **Table 1**). The mean relative *M. haemomuris* genome copy concentration 8 dpi of well A2 appeared higher when compared to 0 dpi (**Figure 44**) but this difference was within the % CV of the assay at the measured relative *M. haemomuris* copies, as previously determined 5.3.1.1. This was of interest as it showed a different trend in *M. haemomuris* genome copies per µl medium, especially as well A2 contained differentiated murine stem cells and stem cell factors at different concentrations, as well as transferrin, that had not been added previously. Well A2 was maintained for 12 additional days of partial media replacement and passaging of growing stem cells (dilution factor accounted for) but did not show an increase, but a decrease, in mean *M. haemomuris* genome copies per µl medium over time.

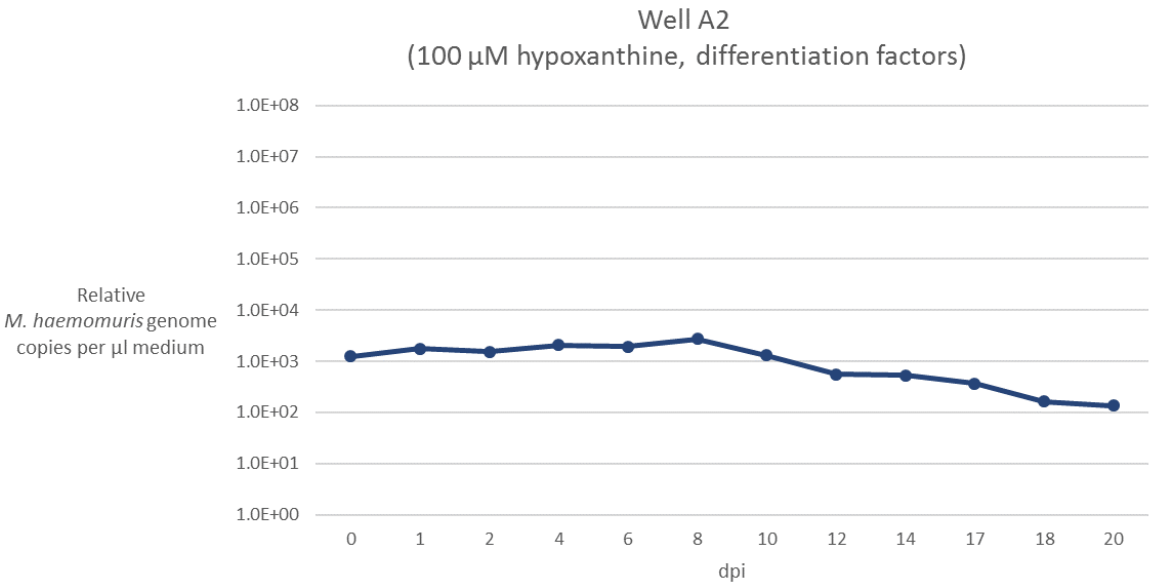


Figure 44 *M. haemomuris genome copies during in vitro cultivation study 1, showing amplification plots of culture well A2 after inoculation of 160µl M. haemomuris-infected mouse blood into 1840µl of prepared medium A2. Days post inoculation are plotted on the x-axis and relative haemoplasma genome copies per µl medium are plotted on the y-axis. Well A2 was subject to partial media replacement daily and passaged (diluted) 1:1 at 2, 4, 6, 8 and 10 dpi. Dilution factors were accounted for before creating the plots. Dpi, days post inoculation.*

Supernatant samples of 100µl, collected at 2 dpi from all wells of *in vitro* cultivation study 1 were subject to DNA extraction and *M. haemomuris*-specific qPCR to assess for potential growth within the media but showed negative or barely detectable (<100) genome copies per µl medium (data not shown). Supernatant was hence not further investigated in subsequent studies.

The sentinel mice S1 and S2, inoculated at 8 dpi of culture wells, using the contents of well A2, after sampling and passaging (inoculation dose per mouse: 1.50E+05 *M. haemomuris* copies) did not establish *M. haemomuris* infection during the observation period of 14 days (**Figure 45**).

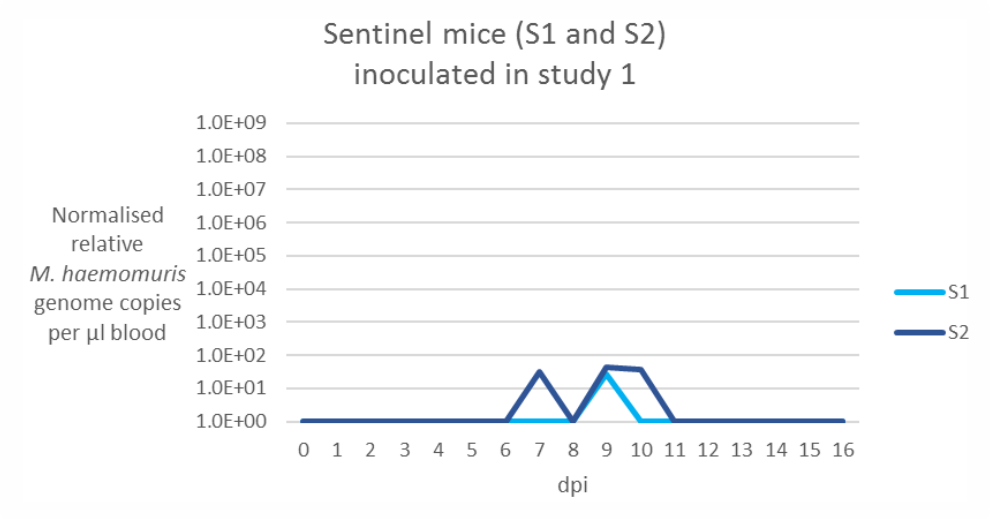
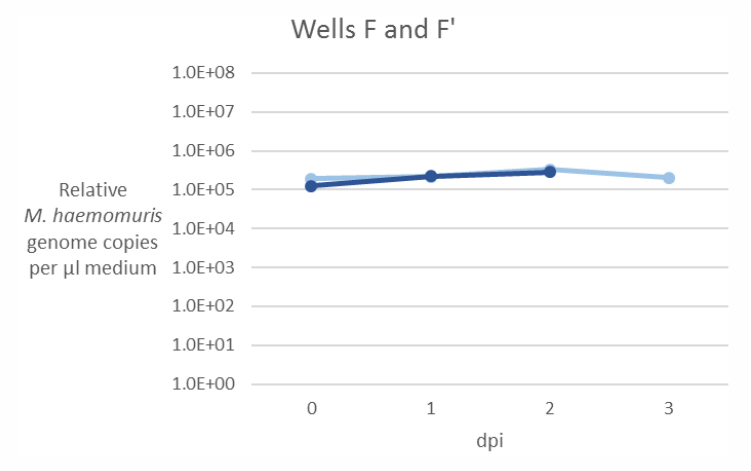
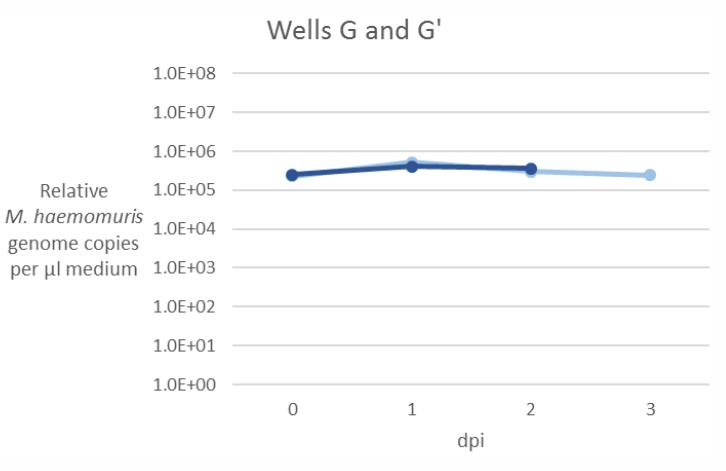


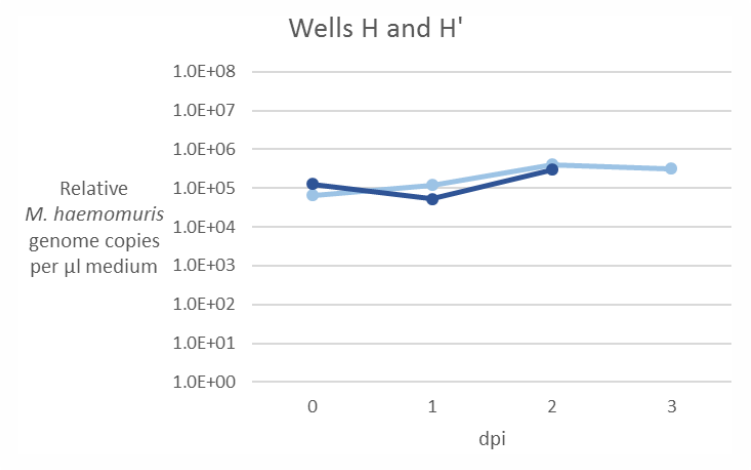
Figure 45 *M. haemomuris genome copies following inoculation of sentinel mice S1 and S2 from in vitro culture well A2 at 8 dpi of culture wells. Days post inoculation (of mice) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl blood are plotted on the y-axis. Lines represent individual mice. Blood sampling occurred once daily.*

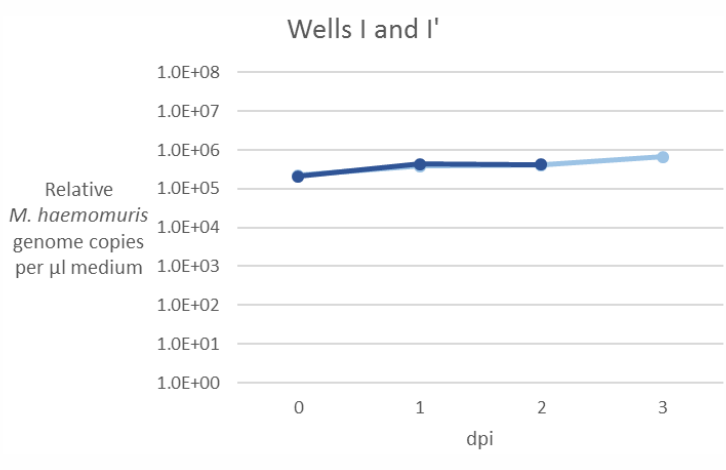
5.3.1.4 Results of in vitro cultivation study 2 using pre-peak infected blood, splenocyte suspensions, differentiated erythroblasts, whole blood and erythrocyte suspensions

There was no growth detected in any of the 500µl wells inoculated during *in vitro* cultivation study 2 (5.2.2.7) with *M. haemomuris*-infected blood collected from a C57BL/6 mouse at 3 dpi (pre peak phase) and combinations of differentiated murine stem cells, stem cell supplements including transferrin and additional hypoxanthine (**Figure 46**). The mean relative *M. haemomuris* genome copies per µl culture medium at 3 dpi of culture wells were not higher (as defined by the assay's limit of quantification) when compared to *M. haemomuris* genome copies in the same medium at 0 dpi for all wells, which would have defined *in vitro* growth. However, *M. haemomuris* genome copies per µl medum were still consistently higher at 3 dpi compared to 0 dpi in wells containing media compositions H, I, J and K (**Figure 46**). This was of interest, as media H, I and J contained uninfected mouse blood or washed mouse erythrocytes, but medium K did not contain any cellular additives. Wells X and X' (see **Figure 46**, **bottom right corner**) showed significant haemolysis following pipetting during the inoculation and sampling/resuspension procedures, and a drop in *M. haemomuris* genome copies per µl medium over 12 and 24 hours post inoculation was attributed to this. The handling procedures of the subsequent studies were thus amended to ensure haemolysis, and the shear stress of cells/haemoplasmas, was minimised by replacing the centrifugation/pipetting steps with gentle rocking of plates during mixing and resuspension steps.


Wells F and F'
Relative M. haemomuris genome copies per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
dpi


Wells G and G'
Relative M. haemomuris genome copies per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
dpi


Wells H and H'
Relative M. haemomuris genome copies per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
dpi


Wells I and I'
Relative M. haemomuris genome copies per µl medium
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
1
2
3
dpi

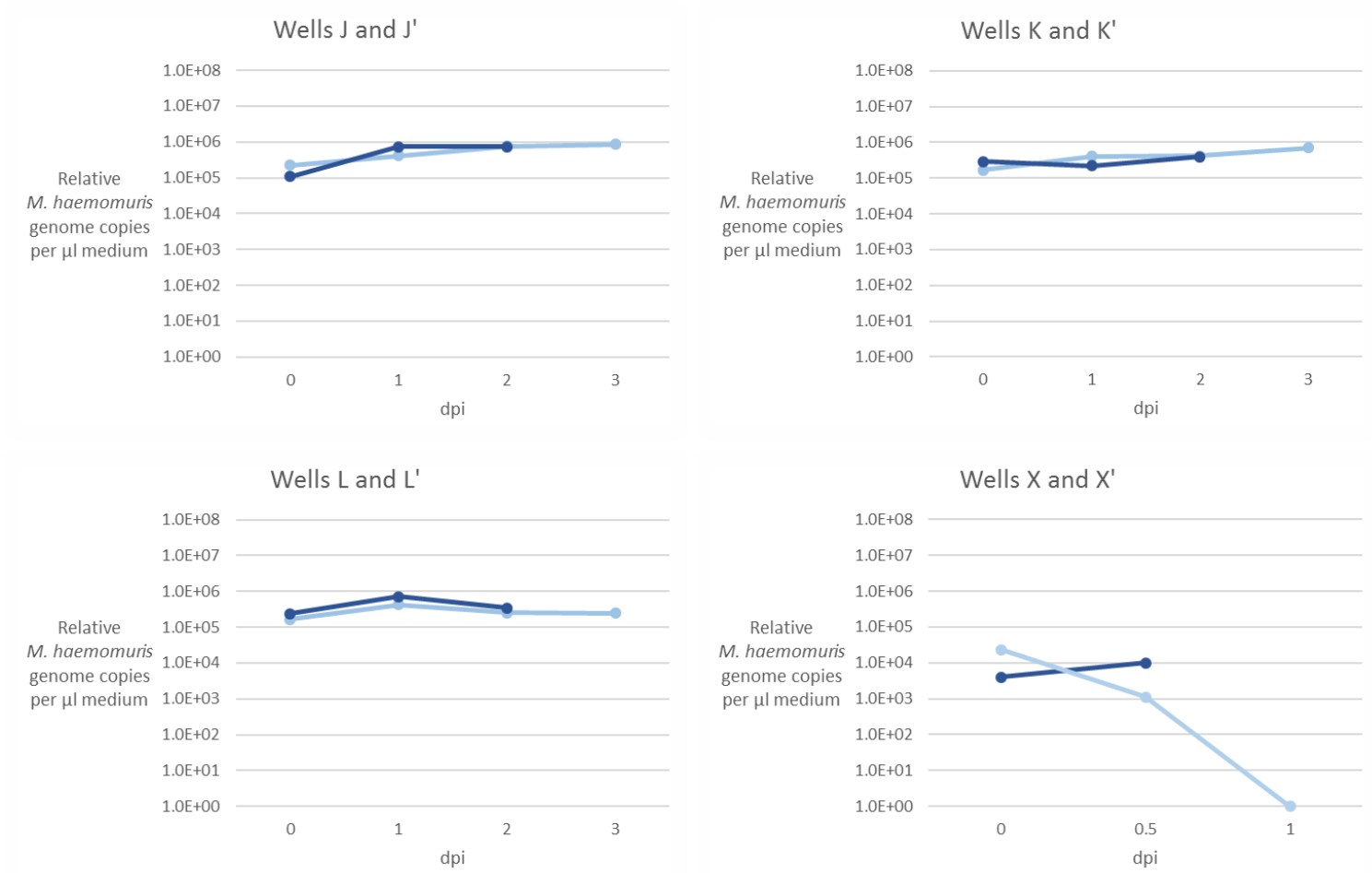


Figure 46 *M. haemomuris genome copies during in vitro cultivation study 2, showing amplification plots of culture wells F and F', G and G', H and H', I and I', J and J', K and K', L and L' as well as X and X' after inoculation of 50µl M. haemomuris-infected mouse blood into 450µl of prepared media F, G, H, I, J, K, L and X. Media compositions are given in* ***Table 48****. Days post inoculation (dpi) are plotted on the x-axis and relative haemoplasma genome copies per µl medium are plotted on the y-axis. Connected dots of the same colour represent individual culture wells: Apostrophe-labelled wells, terminated and inoculated into mice at 2 dpi (0.5 dpi for medium X) are indicated by darker blue colouring. Wells terminated at 3 dpi (1 dpi for medium X) being indicated by lighter blue colouring of lines. Wells were subject to partial media replacements daily and sampled every day. Dilution factors (through sampling) were accounted for before creating the plots.*

The sentinel mice (S3, S4) inoculated at 12 hours post inoculation of culture well X' and 24 hours post inoculation of culture well X (see **Figure 46**, **bottom right corner**) only established typical *M. haemomuris* infection kinetics (as defined by observations in Chapters 3 and 4, e.g. **Figure 21** and **Figure 32**) when inoculated with the 12-hour-culture from well X'. The inability of the inoculum derived from culture well X at 24 hours post inoculation to establish infection was attributed to declining *M. haemomuris* genome copies per µl culture medium coinciding with haemolysis occurring in the same well. Sentinel mice S5, S6, S7 and S8, inoculated from pooled duplicate cultures at 2 dpi (see **Table 49**) all successfully become infected with *M. haemomuris,* and the infection kinetics plots for these sentinel mice are shown in **Figure 47**. The result that sentinel mouse S7 (inoculated from well K') did not show any difference in infection kinetics was of interest as well K' did not contain any cellular additives. This means that, for *in vitro* maintenance of *M. haemomuris* over two days, the addition of uninfected blood or other cellular components (such as stem cells) was not required to maintain *M. haemomuris* viability.

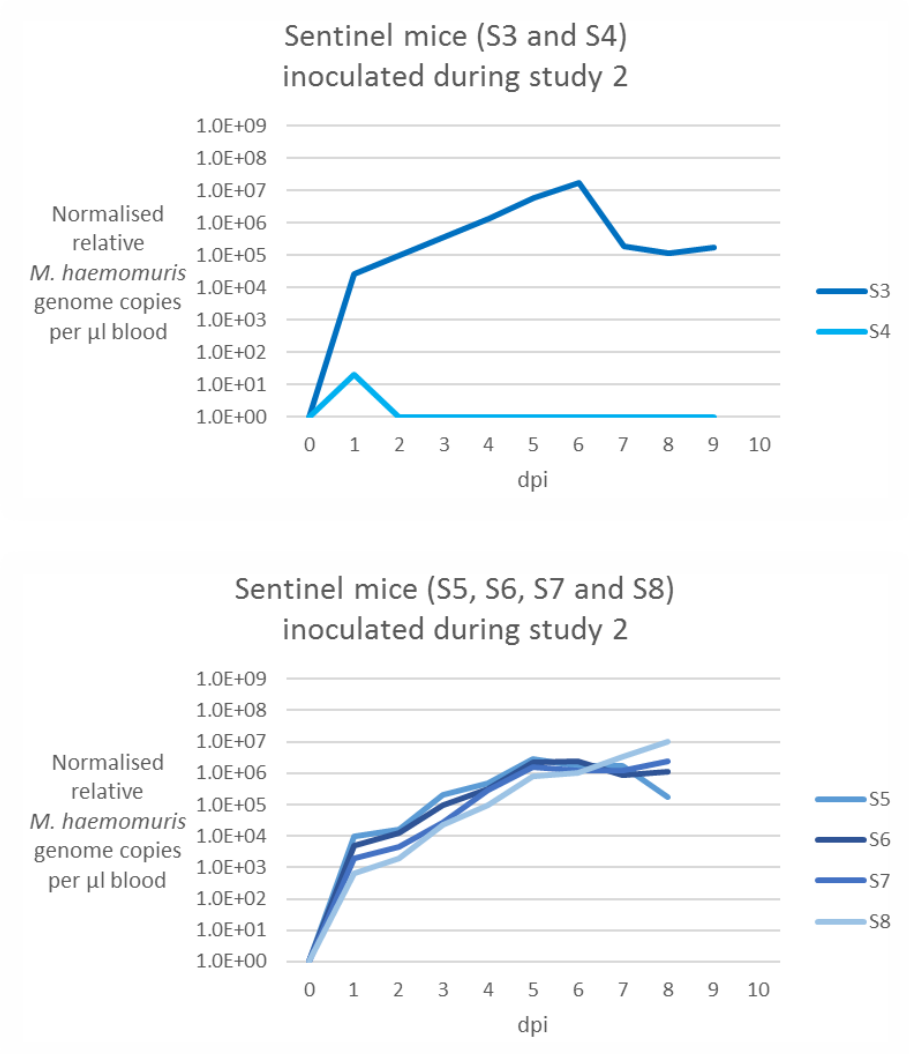


Figure 47 *M. haemomuris genome copies following inoculation of sentinel mice inoculated during in vitro cultivation study 2. Media composition is given in* ***Table 48****. Sentinel mice S3 and S4 (upper panel) were inoculated from in vitro culture well X' at 12 hours (S3) and well X at 24 hours (S4) post inoculation of culture wells. Sentinel mice S5-S8 (lower panel) were inoculated from culture wells G'/I'/L' (S5), H'/J' (S6), K' (S7) and F' (S8) at 2 dpi of culture wells. Days post inoculation (dpi; of mice) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines). Blood sampling occurred once daily but was discontinued ahead of the 14-day monitoring period as infection kinetics became apparent.*

5.3.1.5 *Results of in vitro cultivation study 3 (viability study) using StemPro™-34 without cellular supplementation*

The sentinel mice S9 and S10, inoculated at 2 and 4 dpi of culture wells, using the contents of wells V2 and V4 after daily partial media replacement and careful handling to try and reduce haemolysis, established *M. haemomuris* infection during the observation period of 16 days (**Figure 48**). Sentinel mouse S10 (black dotted line in **Figure 48**) was found dead at 9 dpi with no other overt signs visible other than splenomegaly on post-mortem examination (splenic weight 1.92% bodyweight; normal < 0.4%). The sentinel mice S11, S12 and S13, inoculated at 6, 8 and 10 dpi of culture wells V6, V8 and V10 did not successfully establish infection during the observation period of 16 days. Sentinel mouse S11 yielded normalised relative *M. haemomuris* genome copies per µl blood of 100-1000 copies on two occasions, but both of these positive results were flanked by negative results, including for the rest of the observation period, thus the typical infection kinetics of *M. haemomuris* growth were not seen. These results of successfully infected sentinel mice are of interest as it suggests that *M. haemomuris* remained viable for at least 4 days outside of the host under the explored *in vitro* conditions. No *M. haemomuris* genome copies per µl media were available from study 3 as it aimed to keep cell disturbance at minimum. Media replacement was followed by gentle horizontal rotation of the plates to resuspend sedimented cells in culture media and inoculation was followed by gentle horizontal rotation of the plates to mix the blood and culture media and further pipetting of the mixture was avoided to minimise haemolysis for all subsequent studies.

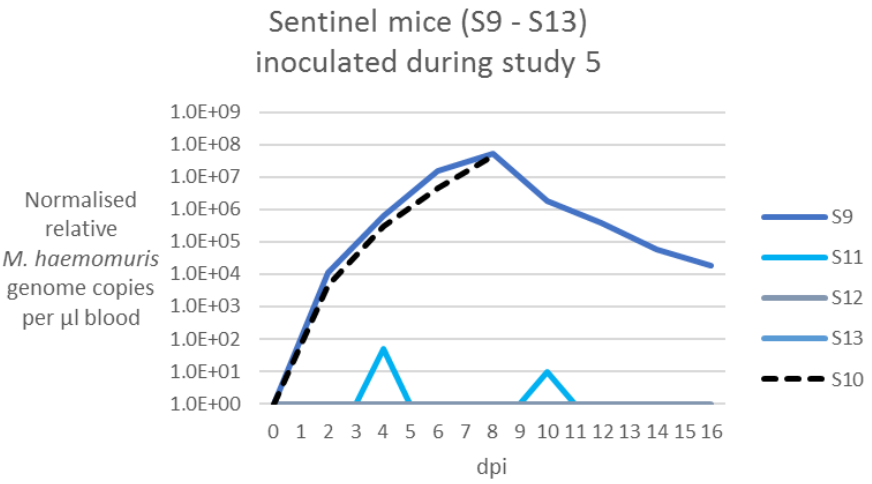


Figure 48 *M. haemomuris genome copies in the sentinel mice inoculated during in vitro cultivation study 3 (viability study) using StemPro™-34 without cellular supplementation (medium V, see* ***Table 50****). Days post inoculation (dpi; of mice) are plotted on the x-axis and M. haemomuris genome copies per µl of blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines); blood sampling occurred once daily. Mice were monitored daily for 16 days to determine successful or unsuccessful M. haemomuris amplification. Sentinel mouse S10 (black dotted line) was found dead at 9 dpi. Sentinel mouse S11 (grey line) did yield normalised relative M. haemomuris genome copies per µl blood of 100-1000 copies at two occasions but this was not flanked by typical infection kinetics.*

5.3.1.6 *Results of in vitro cultivation study 4 (RNA study) using StemPro™-34 without cellular supplementation*

In vitro cultivation study 4 aimed at providing a methodology of detecting declining *M. haemomuris* viability in culture media that did not involve the use of sentinel mice. The infection kinetics curves of *M. haemomuris* genome copies (DNA; representing copies of the *M. haemomuris* 16S rRNA gene) per µl blood, using the previously described qPCR (see 3.2.2.3.2) and 16S rRNA copies (RNA) per µl blood after DNA digestion and RT-qPCR, over the course of two weeks in a 13-week-old mouse are

shown in **Figure 49**. No DNA was detectable in samples following DNase digestion (data not shown), confirming a successful DNA removal.

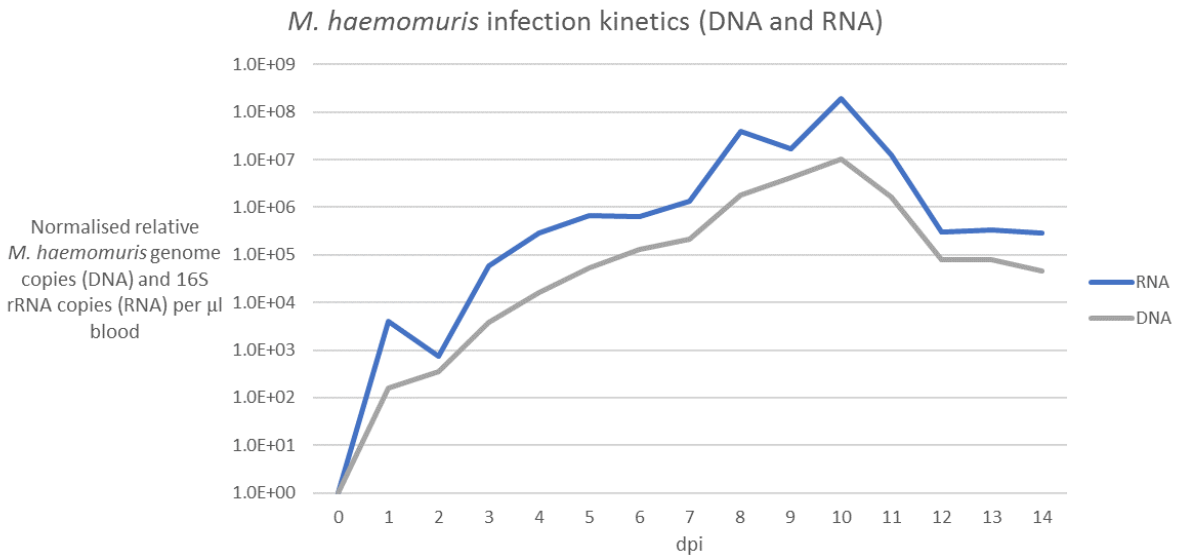


Figure 49 *M. haemomuris infection kinetics curves in one 13-week-old SPF, female C57BL/6 mouse after inoculation with M. haemomuris. Days post inoculation (dpi) are plotted on the x-axis and normalised relative M. haemomuris genome copies (DNA) per µl of blood are plotted on the y-axis as a grey line. Normalised relative* M. haemomuris *16S rRNA copies (RNA) per µl of blood are plotted as a blue line.*

Two identical wells, inoculated with blood containing 7.84E+06 *M. haemomuris* genome copies per µl blood, sourced at 7 dpi, were monitored for 3 and 7 days before inoculation of well contents into two sentinel mice. Relative *M. haemomuris* genome copies (DNA; representing copies of *M. haemomuris* 16S rRNA gene) per µl of medium and relative *M. haemomuris* 16S rRNA copies (RNA) per µl of medium over the 3- and 7 day incubation period of wells W1 and W2 with partial media replacement are shown in **Figure 50**. An increase in *M. haemomuris* genome copies per µl medium over two days following inoculation of culture wells was observed, representing putative *in vitro* growth for the first time. In contrast to earlier studies, *M. haemomuris* genome copies showed over a 20-fold increase (8.88E+04 genome copies per µl medium to 1.96E+06 genome copies per µl medium) over the first 3 days of *in vitro* conditions, before then showing a gradual decline.

Relative *M. haemomuris* 16S rRNA copies per µl of medium mirrored the DNA results but because *M. haemomuris* growth was observed for the first time, work using RT-qPCR was discontinued in favour of optimising and replicating *M. haemomuris* growth under *in vitro* conditions. Detection of RNA had aimed mainly at assessing *M. haemomuris* viability under the assumption that no growth would occur.

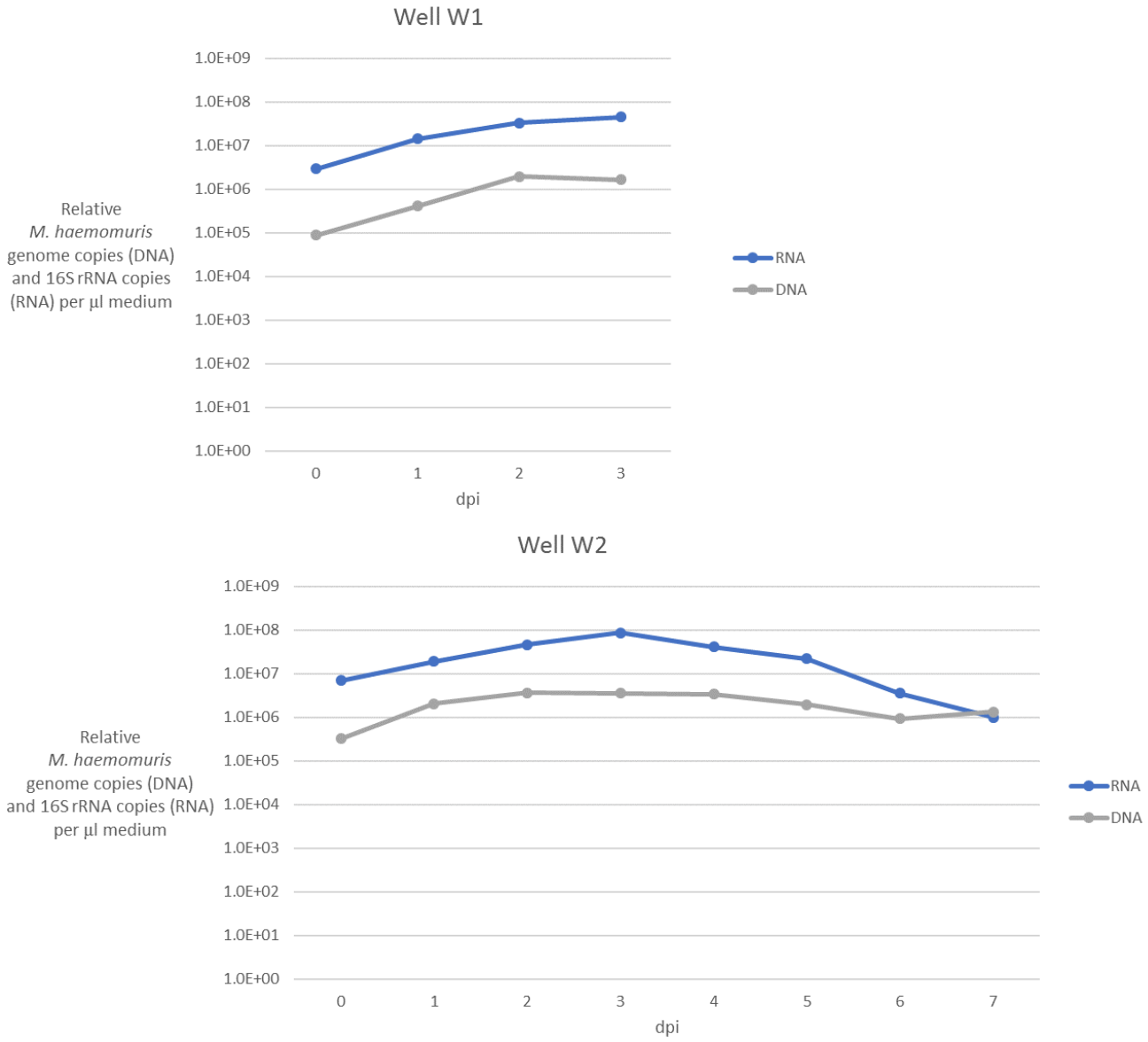


Figure 50 *M. haemomuris genome copies during in vitro cultivation study 4, showing amplification plots of culture wells W1 (upper panel) and W2 (lower panel) after inoculation of 50µl M. haemomuris-infected mouse blood into 450µl of prepared medium of identical composition. Medium composition W is given in* ***Table 52****. Days post inoculation (dpi) are plotted on the x-axis and relative M. haemomuris genome copies (DNA) per µl of medium as well as relative M. haemomuris 16S rRNA copies (RNA) per µl of medium are plotted on the y-axis. Connected dots of the same colour represent individual culture wells, grey lines represent DNA (M. haemomuris 16S rRNA gene copies via conventional qPCR) and blue lines represent RNA (*M. haemomuris *16S rRNA copies via RT-qPCR following DNA digestion). Wells were subjected to partial media replacements daily and sampled every day. Dilution factors (through sampling of 10µl per day) were accounted for before creating the plots.*

The sentinel mice inoculated at 3 dpi with the contents of well W1 (sentinel mouse S14) and at 7 dpi with contents of well W2 (sentinel mouse S15) showed evidence of establishment of *M. haemomuris* infection, but only the sentinel mouse S14 showed typical infection kinetics (**Figure 51**). Sentinel mouse S15 (dark blue line in **Figure 51**) exhibited similar infection kinetics to what had been observed in protective immunity (i.e. lower and fluctuating *M. haemomuris* genome copies per µl blood without a clearly defined peak; see Chapters 3 and 4, e.g. **Figure 23**and **Figure 35**). Due to time restrictions in the experiment duration the infection kinetics of sentinel mouse S15 could not be followed for longer. This inability to establish typical infection kinetics in the sentinel mouse S15 coincided with a drop of *M. haemomuris* 16S rRNA copies per µl medium below the level of *M.*

haemomuris genome copies (DNA) per µl medium suggesting drastically reduced *M. haemomuris* viability in the obtained inoculum (well W2 at 7 dpi, see **Figure 50, lower panel**) despite comparable inoculum doses (*M. haemomuris* genome copies) received by both sentinel mice (**Table 53**).

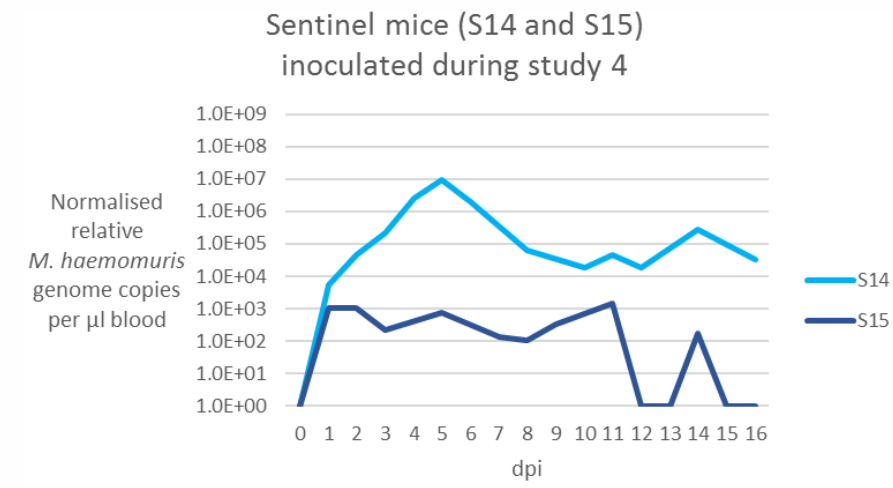


Figure 51 *M. haemomuris genome copies following inoculation of sentinel mice inoculated during in vitro cultivation study 4. Sentinel mice S14 and S15 were inoculated from in vitro culture well W1 at 3 dpi (S14) and well W2 at 7 dpi (S15) of culture wells. Days post inoculation (of each mouse although inoculations did not happen at the same day) are plotted on the x-axis and normalised relative haemoplasma genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines).*

5.3.1.7 Results of in vitro cultivation study 5 (purine/pyrimidine study) using nucleobases, nucleotides, single-stranded DNA and whole blood supplementation

This study aimed to determine whether *M. haemomuris* organisms exhausted nucleobases, nucleosides, nucleotides, single-stranded DNA as putatively needed to synthesise DNA and RNA under the *in vitro* conditions previously tested in sections 5.3.1.3 to 5.3.1.6. Media were prepared as described in **Table 54** to contain a control (N), a nucleobase/nucleoside/nucleotide mixture (NUCLEO), single-stranded Salmon sperm DNA (SALMON) and fresh whole blood replacement at 3 dpi of culture wells (BLOOD). Relative *M. haemomuris* genome copies per µl over the 8-day incubation period with partial media replacement and fresh blood replacement for well BLO2 are shown in **Figure 52**.

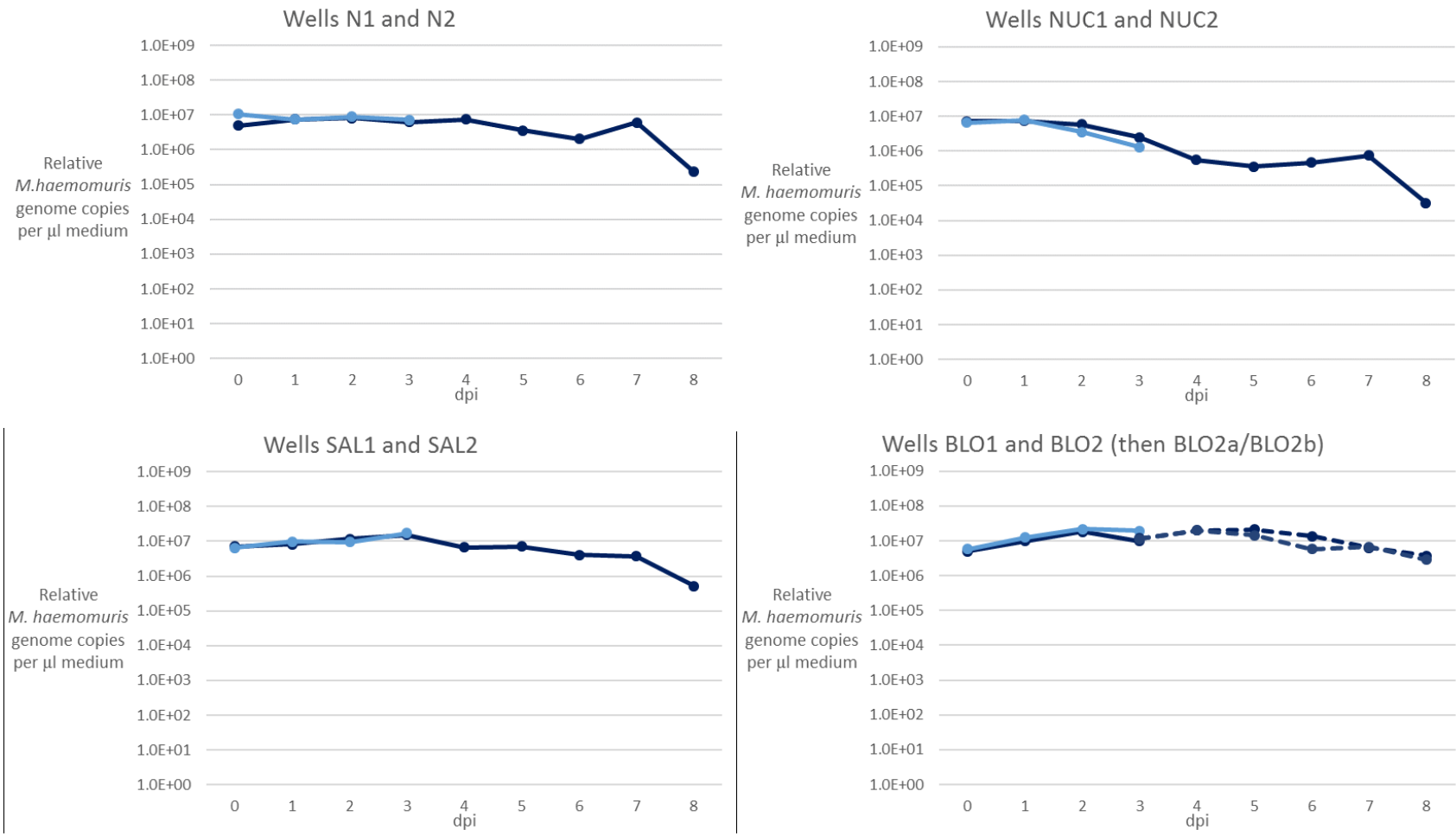


Figure 52 *M. haemomuris genome copies during in vitro cultivation study 5, showing amplification plots of culture wells containing media N (N1/N2), NUCLEO (NUC1/NUC2), SALMON (SAL1/SAL2) and BLOOD (BLO1 and BLO2a/BLO2b after splitting well BLO2 in two wells and inoculation of 50µl M. haemomuris-infected mouse blood into 450µl of the newly prepared well). Medium composition is given in* ***Table 54****. Days post inoculation (dpi) are plotted on the x-axis and relative M. haemomuris genome copies (DNA) per µl per µl medium are plotted on the y-axis. Connected dots of the same colour represent individual culture wells. Light blue lines represent wells discontinued at 3 dpi (well names ending in '1') and inoculated into sentinel mice. Dark blue lines represent wells continued until 8 dpi. Well BLO2 was split in two identical wells and reconstituted in media containing fresh blood at 3 dpi which is indicated by the separation of dashed lines at 3 dpi in the bottom right panel and illustrated in more detail in* ***Figure 54*** *Well BLO2a was inoculated into a sentinel mouse at 8 dpi. Wells were subject to partial media replacements daily and sampled every day. Dilution factors (through daily sampling of 10µl and the 1:10 dilution at 3 dpi) were accounted for before creating the plots.*

Growth was only apparent in the wells containing media BLOOD and SALMON over the first three dpi of the culture wells and not in the wells containing media NUCLEO and N, despite medium N comprising identical composition to medium BLOOD until the later wells were split and reconstituted with fresh mouse blood (media compositions see **Table 54**). The failure to amplify *M. haemomuris* in medium N was attributed to potential contamination of the cultures as evidenced from adhered cell layers (see **Figure 53, left panel**). This adhered cell layer could only be resuspended by pipetting, and this was avoided in the remaining wells over concerns of inducing haemolysis. Haemolysis was evident in well N2 at subsequent days of the experiment (see **Figure 53, right panel**).

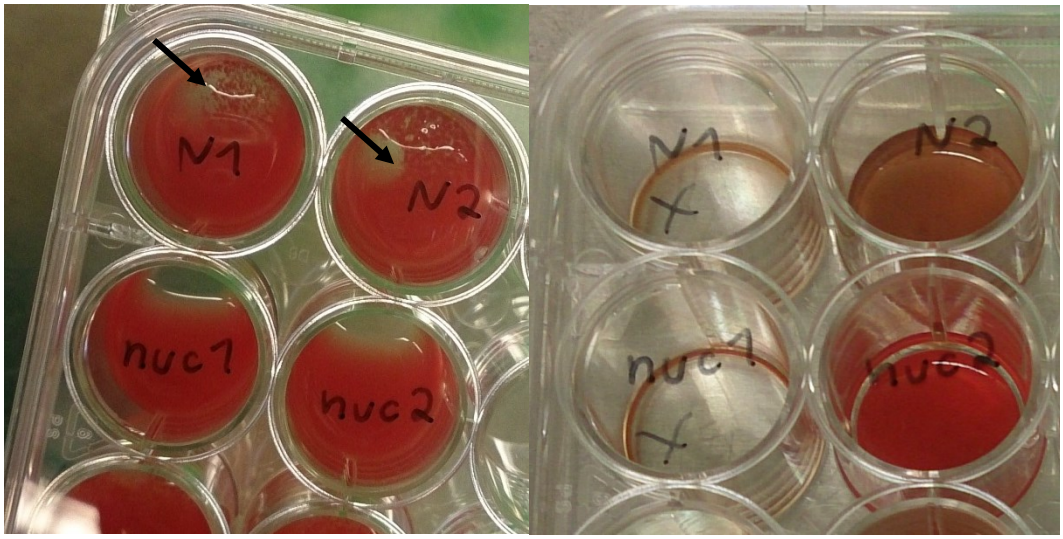


Figure 53 *Culture wells N1/N2 and NUC1/NUC2 at 3 days post inoculation of culture wells (left panel) showing an adherent layer of cells as red specks in the top half of the wells N1/N2 (indicated by arrows) that was not apparent in the remaining wells. The right panel shows the same wells at 5 dpi, after N1 and NUC1 had been inoculated into sentinel mice and haemolysis became apparent in well N2.*

After the initial three days of the experiment, growth could only be sustained in wells BLO2a and BLO2b after splitting the well contents and reconstituting them in fresh media containing uninfected mouse blood (the wells are represented by dashed dark blue lines in **Figure 52, bottom right panel**). For illustration purposes, and to highlight growth on a logarithmic scale, the *M. haemomuris* amplification in wells BLO2a and BLO2b is shown on a smaller scale in **Figure 54** below.

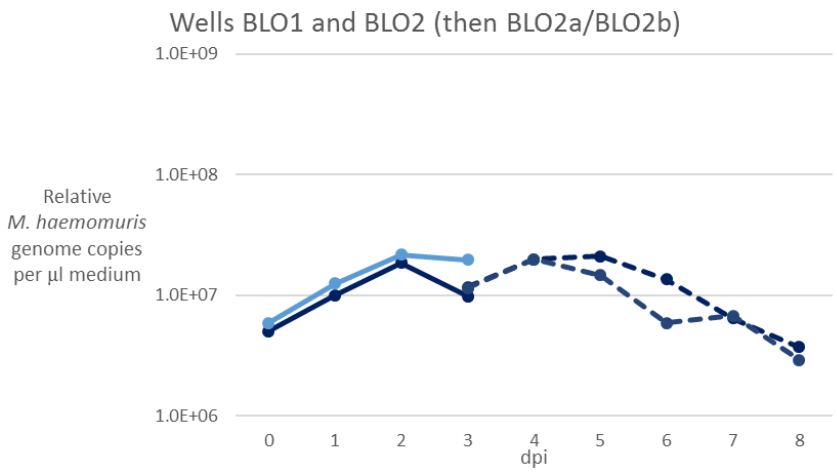


Figure 54 *M. haemomuris genome copies during in vitro cultivation study 5, showing amplification plots of culture wells BLO1 (light blue line) and BLO2 (dark blue line) and BLO2a/BLO2b after well BLO2 was split into two wells, supplemented with fresh mouse blood at 3 dpi of culture wells. Data to create this graph has already been shown in* ***Figure 52*** *but is presented with an optimised scale to highlight the observed growth pattern of* M. haemomuris *over time. Days post inoculation (dpi) are plotted on the x-axis and relative haemoplasma genome and relative M. haemomuris genome copies (DNA) per µl medium are plotted on the y-axis. Connected dots of the same colour represent individual culture wells. Light blue lines represent wells discontinued at 3 dpi and inoculated into sentinel mice. Dark (and subsequently dashed) blue lines represent wells continued until 8 dpi.*

All sentinel mice, inoculated at 3 dpi of culture wells (N1, NUC1, SAL1 and BLO1) showed typical *M. haemomuris* infection kinetics (see sentinel mice S16 to S19 in **Figure 55**). Sentinel mouse S16 (dashed black line in **Figure 55**) was found dead at 7 dpi (of the mouse) with no other overt signs visible other than splenomegaly on post-mortem examination (splenic weight 1.89% bodyweight; normal <0.4%). Sentinel mouse S20, inoculated from *in vitro* culture well BLO2a at 8 dpi of the culture well, showed a prolonged lag-phase but ultimately amplified *M. haemomuris* to similar genome copies per µl blood (>1.0E+07/µl) as it is seen during primary infection (see light blue line in **Figure 55**).

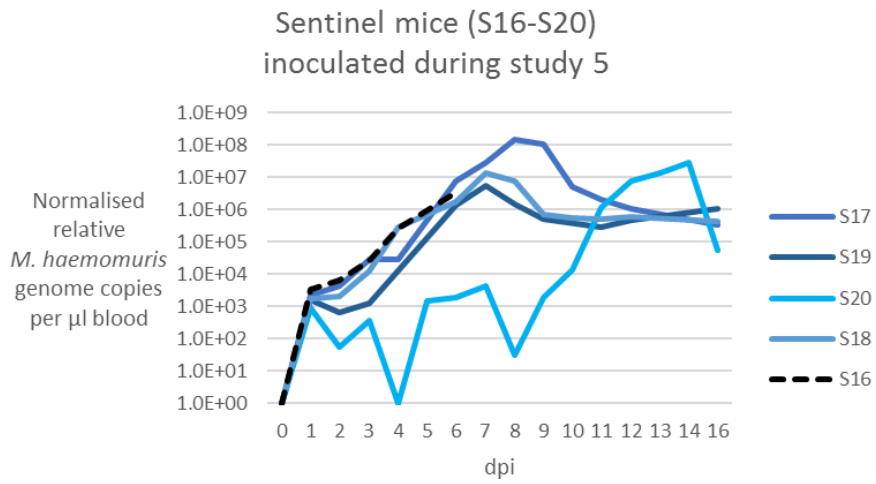


Figure 55 *M. haemomuris genome copies following inoculation of sentinel mice inoculated during in vitro cultivation study 5. Sentinel mouse S16 (dashed black line) was inoculated from in vitro culture well N1 at 3 dpi of the culture well and found dead at 7 dpi. Sentinel mice S17, S18 and S19 were inoculated from* in vitro *culture wells NUC1, SAL1 and BLO1 at 3 dpi of the culture wells and showed typical* M. haemomuris *infection kinetics. Sentinel mouse S20 was inoculated from* in vitro *culture well BLO2a at 8 dpi of the culture well and showed a prolonged lag-phase but ultimately* amplified M. haemomuris to *typical levels of primary infection. Days post inoculation (of each mouse although inoculations did not happen at the same day) are plotted on the x-axis and normalised relative haemoplasma genome copies per µl blood are plotted on the y-axis. Lines represent individual mice (mice differentiated by colouring of the lines).*

The retained viability of the contents of well BLO2a, in conjunction with the observed growth following fresh blood supplementation at 3 dpi of culture well BLO2, led to the conclusion that *M. haemomuris* viability was prolonged from that previously reported (4 dpi) in 5.3.1.5 to 8 dpi, likely due to the culture media being replaced and supplemented with fresh mouse blood at 3 dpi.

5.3.1.8 Results of in vitro cultivation study 6 (continuous culture pilot) using serial media expansions and blood replacement

Based on the results of *M. haemomuris* growth in the preceding experiment it was decided to replicate similar experimental conditions comprising of media replacement combined with addition of fresh mouse blood at 2 dpi of culture wells as *M. haemomuris* genome copies per µl culture medium started to decline after 2 dpi of consecutive culture (**Figure 54**). Upon passaging of 50µl of infected culture into 450µl of new media containing 50 µl of fresh mouse blood at 2 dpi, *M. haemomuris* showed a 39-fold increase from 5.03E+06 to 1.95E+08 *M. haemomuris* genome copies per µl culture medium (**Figure 56**).

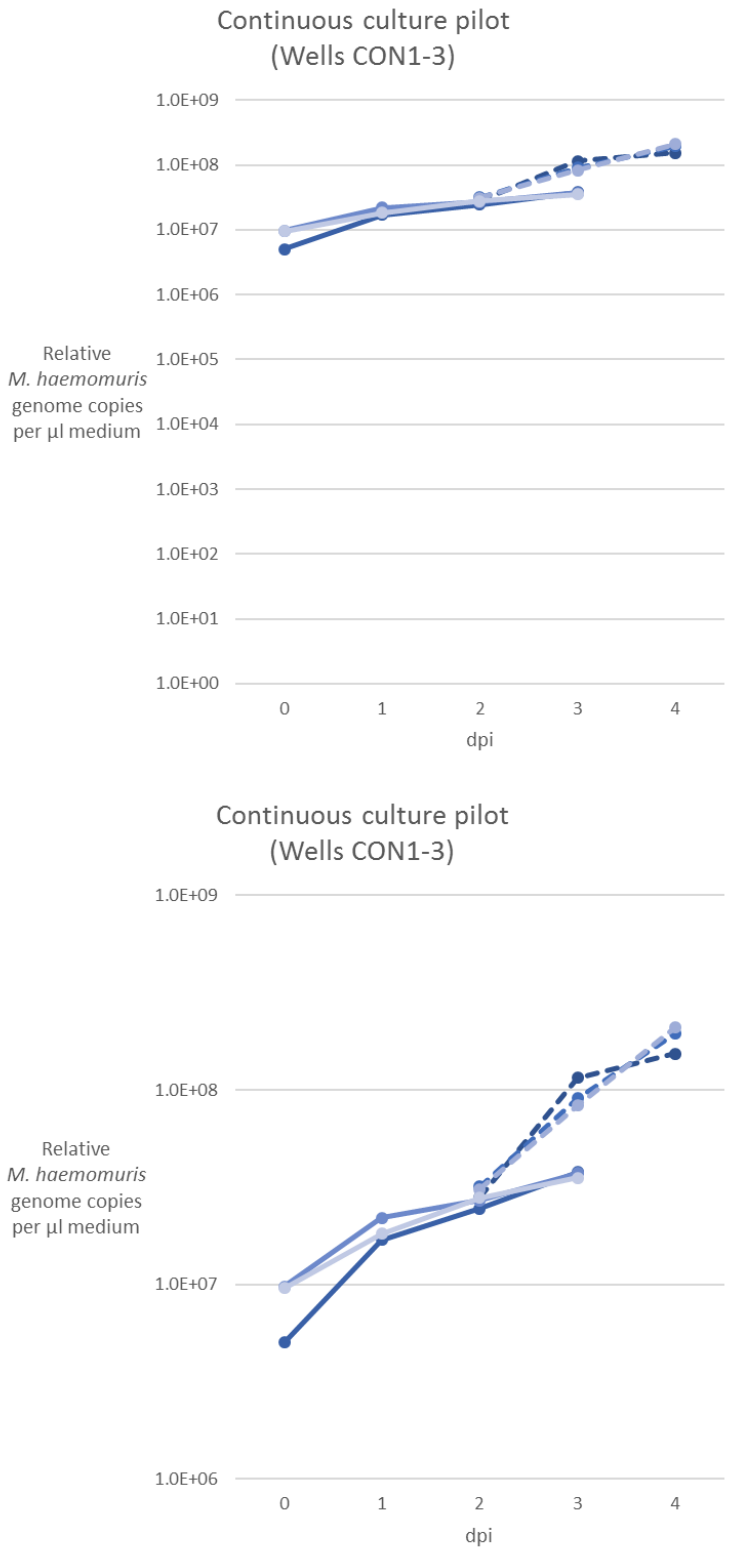


Figure 56 *M. haemomuris genome copies during in vitro cultivation study 6, showing amplification plots of culture wells CON1, CON2 and CON3 (solid blue lines) before and after passaging them into recipient wells supplemented with fresh mouse blood at 2 dpi of culture wells (dotted blue lines). Medium composition (CONT) is given in* ***Table 56****. Days post inoculation (dpi) are plotted on the x-axis and relative haemoplasma genome and 16S rRNA copies per µl medium are plotted on the y-axis. Connected dots of the same colour represent individual culture wells. Dilution factors have been accounted for before creating the plots. The lower panel is based on the same data as the upper panel but uses a smaller scale to illustrate M. haemomuris in vitro growth.*

The sentinel mouse (S21) inoculated at 4 dpi of culture wells with contents of the recipient well from CON1 (light blue dashed line in **Figure 56**) successfully amplified *M. haemomuris* and showed typical infection kinetics as shown in **Figure 57**.

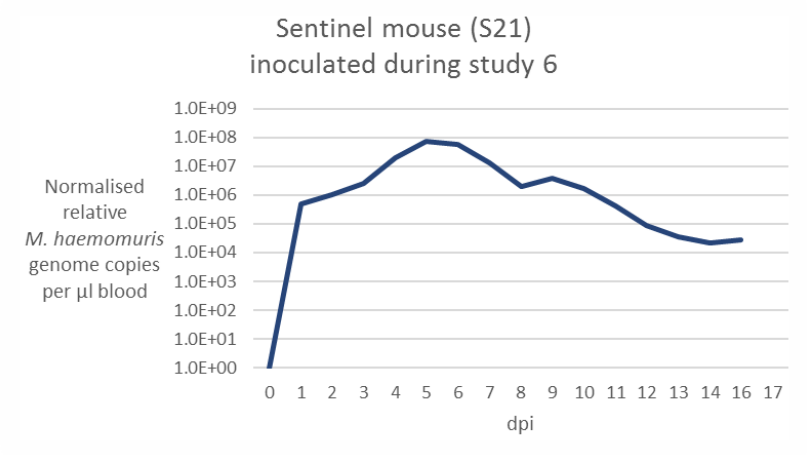


Figure 57 *M. haemomuris genome copies following inoculation of one sentinel mouse inoculated during in vitro cultivation study 6 from the in vitro culture well that had been the recipient well of culture well CON1. Inoculation of the sentinel mouse happened at 4 dpi of the original culture well. Days post inoculation (dpi) are plotted on the x-axis and normalised relative M. haemomuris genome copies per µl blood are plotted on the y-axis.*

5.4 DISCUSSION

With the cultivation pilot studies described in this chapter, we have managed to fulfil two of four of Robert Koch's postulates (412) for a haemoplasma species, namely *M. haemomuris.* The first postulate calls for the pathogen to always be present in cases of the disease, which can be considered already established for the disease haemoplasmosis (11) and has not been investigated in the current study. The second postulate states that a pathogen must be isolated from the host and grown in culture to be called the causative agent of the disease as has been demonstrated in the present study. The third postulate states that the pathogen isolated in culture must cause the disease when inoculated into a healthy, susceptible laboratory animal (405), which is consistent with successful infection of sentinel mice with cultured *M. haemomuris* described in the present study. The fourth postulate states that the newly, experimentally infected host must allow recovery of the same organism which, although assumed to be true, has not been attempted in the current study as no sentinel-mouse-derived *M. haemomuris* inocula were used for subsequent *in vitro* cultivation attempts. Whilst these postulates might be considered incomplete in terms of defining 'causation' by either a non-cultivatable pathogen or a defined community of synergistic pathogens, they still hold relevance for the scientific rigor that goes into their application to investigating novel pathogens and diseases (413).

5.4.1 Successful *M. haemomuris in vitro* maintenance and *in vitro* cultivation

Previous reports on the *in vitro* maintenance of haemoplasmas were incomplete, due to the lack of fulfilment of Koch's third postulate showing viability of the organism (216, 241, 242), or due to the methodology used (i.e. blood smear examination and non-molecular detection techniques for the haemoplasmas as employed by Nonaka, Thacker (241) for *M. suis*) that did not allow repetition of the experiment due to a failure to define the pathogens in question (239, 240). In this chapter we were able to inoculate naïve laboratory mice with *M. haemomuris* that had its viability conserved under *in vitro* conditions for a period of eight days; additionally, we were able to vary the *in vitro* conditions and show not just maintenance of *M. haemomuris* genome copies within the medium but actual growth.

Due to limitations in the volume of available inoculum i.e. *M. haemomuris*-infected mouse blood, that could be obtained at various experimental steps and the limitations in the sample volumes that could be taken from the same culture to monitor for change (e.g. growth) over time, the experimental approach was not always linear in terms of varying only one condition at a time, but focused on creating an environment that favoured the survival of the blood cells in culture. A more stepwise approach, using dilution series of various additives and varying culture conditions for each dilution, might have provided a more straightforward approach. However, serial dilutions of various additives and culture conditions, and exploring all possible variations, were very unlikely to have been possible due to the large mouse blood volumes that would have been needed for all of the experiments, and limitations in the repeated sampling of wells that would have had to have been set up in smaller volume formats (i.e. 96-well plates with 0.1 ml culture volumes per well) to alleviate the limitations in available mouse blood of identical origin. After having established the conditions that favoured *M. haemomuris* maintenance and growth, we have established a baseline from which to improve conditions in future experiments. This baseline will allow for future studies to be performed that vary just single additives and culture conditions in order to refine and better define the growth requirements of *M. haemomuris*; such findings could also inform potentially successful culture conditions for other haemoplasmas of veterinary relevance.

When using automated TNA extraction (Chemagen, see 4.2.2.3.2) to extract nucleic acids from undiluted heparinised mouse blood, the heparin remaining within the blood acts as a potent qPCR

inhibitor (414). In the current study (data not shown) it was found that heparin-induced inhibition of qPCR no longer occurred if less than 2.5% (v/v) heparinised blood was present in the sample used for automated TNA extraction. This consideration of qPCR inhibition led to EDTA-anticoagulated blood samples being used for the cytokine expression and haematology studies in which high-throughput, and thus automated, TNA extraction was required, and samples contained 10% (v/v) heparinised blood. However, concentrations of heparinised blood above 2% (v/v) were not achieved in the culture protocols. It has not yet been determined whether EDTA, heparin or other anticoagulants are more or less likely to interfere with haemoplasma viability or growth. The anticoagulant (li-heparin) used in this chapter was chosen based on anecdotal observations that *M. haemofelis* adherence to erythrocytes is decreased in the presence of EDTA, which could reflect reduced viability in EDTA; published rigorous evidence of this hypothesis is lacking (241). Future experiments should include a range of anticoagulants or immediate (within seconds) high dilutions of donor blood into experimental media to avoid the use of anticoagulants and prevent the blood clotting that occurs with low dilutions of blood in saline or other media (415).

5.4.2 Defining growth parameters for *M. haemomuris* and other haemoplasmas

It is not known which substrates in the media combinations used in the current studies in this chapter allowed *M. haemomuris* to have short-lived growth that were not provided in previous attempts at *M. suis* cultivation by Nonaka, Thacker (241) in Eagle's medium and RPMI-1640, and by Schreiner, Hoelzle (242) in the mycoplasma media SP-4 medium and Hayflick's medium, or in attempts at *M. haemofelis* cultivation by Hicks (216) in RPMI-1640 and the proprietary MEXp (243) media. Because the base medium, StemPro™-34, used in the current studies contains various proprietary nutrient supplements that also specifically favour stem cell proliferation (416) one can speculate that these supplements may have had a positive influence on haemoplasma viability. In contrast, the base medium RPMI-1640 does not contain glucose, nucleotide precursors or fatty acids and hence is dependent on the addition of serum (typically foetal calf serum) to provide these in cultivation studies (417), as used in cultivation experiments for *M. haemofelis* by (216). RMPI-1640 is suitable for cultivation of mammalian lymphocytes but requires them to have considerable metabolic capabilities to synthesise substrates not provided in the basal formulation. Eagle's medium (and modified versions, such as DMEM) is similar to RMPI-1640 and contains amino acids, vitamins and glucose but no fatty acids or other nutrients such as nucleotide precursors or growth factors, potentially required by haemoplasmas, as it was originally developed for a variety of mammalian cell lines of higher metabolic capability than haemoplasmas (418). Mycoplasma media (i.e. SP-4 medium and Hayflick's medium) are richer in nutrients and typically contain yeast extract to provide a source of fatty acids, cholesterol and nucleotides (242). However, haemoplasmas may not have been able to utilise yeast extract when not attached to erythrocytes as some studies suggest haemoplasmas scavenge these molecules directly off the host erythrocyte membrane (116, 419). The latter was suggested by our preliminary results that showed no growth but a rapid decline in *M. haemomuris* genome copies in the presence of overt haemolysis and is supported by the findings by Schreiner, Hoelzle (242) that describe nanotransformation, but not growth, when using these media. Furthermore, our studies found that sources of iron (transferrin) appear to be crucial for haemoplasma maintenance/growth, as we found growth only in culture media compositions containing 0.5mg/ml transferrin. Additionally, nucleotides (or their precursors) may be utilised from single-stranded DNA (see 5.3.1.7), as we found an increase in *M. haemomuris* copies in the culture wells, supplemented with it. However, the role of these additives, including insulin (which is also included in the basic formulation of StemPro™-34, but in none of the other media previously tried with haemoplasma cultures) will have to be explored in future experiments. The role of hypoxanthine is equally unclear despite our preliminary data from a study (data not shown) that

tried to reproduce the results described in 5.3.1.8 but unintentionally (due to human error) used a much lower concentration of hypoxanthine and did not result in *M. haemomuris* growth. However, hypoxanthine is also produced by erythrocytes, so the minimal PCV to support *M. haemomuris* viability and growth is yet to be determined. Hicks (216) supplemented SP-4 and undefined (due to proprietary nature of these culture media) MEXp culture media with non-infected, heparinised feline blood, but it could be that the concentration used, 20µl heparinised blood in 1ml culture wells, was simply too low a concentration of blood to allow for *M. haemofelis* propagation *in vitro*. Ideally, *in vitro* cultivated erythrocytes could be utilised for future cultivation attempts, however the current technology cannot mature erythrocytes beyond the erythroblast stage. Our results that showed no growth in the presence of mouse erythroblast precursors or differentiated erythroblasts (5.3.1.4) appear to be consistent with *M. haemomuris*' inability to infect erythrocyte precursors. We did not attempt to cultivate *E. coccoides in vitro* because our mouse model focused on *M. haemomuris*. Additionally, we do not have whole genome sequences available for *E. coccoides* nor *M. haemomuris*, to allow us to compare and deduce the metabolic abilities of these haemoplasmas. It remains to be explored what factors favoured the ability of *E. coccoides* to be propagated in chicken embryos (9), when similar propagation could not be demonstrated for *M. haemomuris* in later studies using an identical approach (197).

5.4.3 Limitations of the current *in vitro* cultivation system and suggestions for further work using *M. haemomuris in vitro* cultivation

One major limitation of the current *in vitro* cultivation work of *M. haemomuris* is that the growth requirements of *M. haemomuris* have not yet been clearly defined and we have not been able to create a truly continuous *in vitro* cultivation system that would allow for log-fold increases in *M. haemomuris* genome copies per µl media as is seen *in vivo* during experimental infection. It would be beneficial if future experiments concentrated on:

a) defining the growth requirements of *M. haemomuris* by selectively removing additives and comparing increases or decreases in *M. haemomuris* organism numbers to the conditions used to produce *M. haemomuris in vitro* growth in the current study and

b) use serial passaging and media expansion (dilution of primary cultures rather than inoculation of new recipient wells) to allow for growth curves over longer time periods than the 4 days used in the current study and hence allow for increased yields of *M. haemomuris* organisms grown under *in vitro* conditions.

6 DISCUSSION

6.1 SUMMARY

The studies presented within this thesis used a range of techniques to establish and characterise a mouse model of haemoplasma infection. Prior to this work, rodent haemoplasma infections had been described but no model had been established. Additionally, the lack of an *in vitro* cultivation system was a huge barrier to haemoplasma research. Haemoplasmas could only be generated and assessed for viability within their natural (usually larger and more expensive) host species, which further slowed down *in vitro* cultivation attempts. In the presented studies, wild source rodent haemoplasmas were collected and phylogenetically identified. These haemoplasmas were passaged into laboratory rodents and infection kinetics monitored by qPCR. One isolate of *M. haemomuris* was chosen to establish a surrogate model of (particularly feline) haemoplasma infection and cross-protection following *E. coccoides*-infection and protection from re-infection with the same *M. haemomuris* isolate assessed in mice. The finding of partial protection from re-infection was then used to design a larger study to characterise the mouse immune response to naïve *M. haemomuris*-infection and to re-inoculation. Principal component analysis was used to describe the cytokine response in naïve *M. haemomuris* infection and partial protection from re-infection with the same *M. haemomuris* isolate. Additionally, the mouse model enabled generation of viable *M. haemomuris* organisms for *in vitro* cultivation studies, leading to the first ever successful *in vitro* cultivation of a haemoplasma over a period of four days.

6.2 MOUSE MODEL

Initial studies (Chapter 2) sourced viable haemoplasmas from wild rodents and transferred them into laboratory rodents, using DMSO cryopreservation previous used for other haemoplasmas (13, 107, 420). The cryopreservation methods used changed during subsequent studies due to the infection failures seen (Chapter 3) that were attributed to the cytotoxic effects of DMSO on viable haemoplasma genome copy numbers in the inocula. These studies highlighted the need to describe haemoplasma copy numbers based on viability rather than those detected by qPCR, as the latter can be an overestimation. The ideal would be to assess haemoplasma copy numbers by determination of colony forming units, but this requires an *in vitro* cultivation system.

Phylogenetic classification of the wild rodent haemoplasma isolates (Chapter 2) was based on 16S rRNA gene sequences, which may not accurately reflect species-versus-strain separation (outlined in Chapter 1). However, very few rodent haemoplasma sequences are available for non-16S rRNA genes (16, 277, 280, 289), so comparison based on other genes to clearly define novel species was not possible in the current studies.

The ability of *M. haemomuris* to change rodent host species, more specifically from *Apodemus* spp. into *Mus musculus*, has been demonstrated in a previous study using molecular methods (197). Rats however, were not able to amplify this *M. haemomuris* isolate in the same study (197). The presented studies detected short-lived bacteraemia after intraperitoneal inoculation of *M. haemomuris* in one rat. Older reports of infection with different haemoplasma species are difficult to interpret as we now know that haemoplasma infections may have pre-existed in the laboratory animals used in studies (35) and a lack of molecular identification of the haemoplasmas in these studies make it very difficult to confirm that different haemoplasma infections had been established (236, 240). With ongoing reports of novel haemoplasmas in varied host species (**Table 1**, Chapter 1) the host specificity of haemoplasmas may have to be re-evaluated. More sequence data from

previously published isolates and ideally, cross-infection studies are required to define each haemoplasma isolate. Our *M. haemomuris* isolate showed 99% identity on 16S rRNA gene sequence to other *M. haemomuris* isolates, which is said to indicate identical species (19). However, our *E. coccoides* isolate showed only 97% identity on 16S rRNA gene sequencing to previously published sequences, and hence could represent a novel species. On 16S rRNA gene sequencing our *M. haemomuris*-like haemoplasma isolate showed 99% identity to a UK 'vole haemoplasma' (277) and 98% identity to an Israeli gerbil haemoplasma (34), and so it is not clear as to whether this represents a novel species.

Quantitative PCR assays are designed to detect and quantify species-specific gene sequences. Validation of qPCRs should use samples collected from the natural host species to ensure cross-reaction with other pathogens or host DNA sequences does not occur. The novel qPCR assays developed in the presented studies (Chapter 3) were specific for *M. haemomuris* and *E. coccoides* and sensitive, detecting down to one to ten genome copies per reaction. The reliability of the *M. haemomuris* duplex qPCR assay (Chapter 5) was confirmed by its average %CV being less than 0.79% (**Table 13** and **Table 14**, Chapter 5), but %CV was not assessed for the *E. coccoides* qPCR assay. Both the *M. haemomuris* and *E. coccoides* qPCR assays were duplexed with an internal host control qPCR to enable the concurrent detection of mouse DNA, helping to screen for, for example, pipetting errors or qPCR inhibitors. The specificity of species-specific qPCRs does mean that novel haemoplasma species may not be detected when these assays are applied to e.g. prevalence surveys. Such studies should also include generic PCR assays to potentially detect novel haemoplasma species that can then be further characterised by DNA sequencing. The species-specific duplex qPCR assays in the presented studies were used to describe the infection kinetics of *M. haemomuris* and *E. coccoides* (Chapter 3). We were also able to demonstrate that partial protection from re-infection with *M. haemomuris* occurs but that cross-protection from *M. haemomuris* following previous *E. coccoides* infection does not occur. These findings agree with previous studies in cats demonstrating partial protection from re-infection with the same haemoplasma species of *M. haemofelis* (107) and '*Ca*. M. turicencis' (124) and the lack of cross-protection from *M. haemofelis* following previous '*Ca.* M. turicensis' infection (295).

The presented studies (Chapter 4) represent the first steps in the validation of a mouse haemoplasma model that can be used for translational studies (drawing conclusions from mouse haemoplasma infection and host response to investigate the pathophysiology and prevention of feline and porcine haemoplasmosis) to assess protective immunity to infection and haematological changes during infection. The haematological data we generated in our studies was not complete as the small sample sizes and limited sampling days in the study meant that we may have missed troughs and/or peaks in certain parameters. Nonetheless, anaemia developed, and six out of 214 haemoplasma-inoculated mice died at peak parasitaemia, so this mouse haemoplasma model would likely allow for future studies of potential therapeutic interventions and haemoplasma virulence factors. However, the lack of ante-mortem blood samples in the mice that died means that we are unable to confirm whether severe anaemia was the cause of death in those mice.

The cytokine expression study (Chapter 4) described cytokine signatures associated with naïve haemoplasma infection and after re-inoculation with the same *M. haemomuris* isolate. A Th17-driven immune response, that is often seen in autoimmunity and may occur with infections, inappropriately handled by Th1 and Th2 pathways (375), was identified in naïve infection that was absent during partial protection. Hence the Th17-driven immune response may be associated with the development of clinical haemoplasmosis. Unfortunately, a lack of samples available from the mice that died means that we could not assess whether dysregulation or an inappropriate immune

response was a contributor to clinical severity and mortality. The similarities in the cytokine response (e.g. increased proinflammatory cytokines IL-6 and TNF-α following re-inoculation) seen in the cytokine expression study (Chapter 4) and following *M. haemofelis*-infection in cats, but not following infection with the less pathogenic '*Ca*. M. turicensis' (124), further helps validate that our mouse model could be used as a translational model for pathogenic haemoplasma infection in cats. Our cytokine signature findings in *M. haemomuris*-infection also could help with the design and assessment of the efficacy of possible vaccines, as cytokine patterns can be used to identify desirable responses to vaccination during vaccine development and before large-scale trials. Further studies are needed to confirm the role of the Th17-pathway in clinical haemoplasmosis and investigate the potential for Th17-induced autoimmunity. Our dataset is limited by its small size and the identified cytokine signatures will need to be confirmed using larger numbers and statistical analysis before being used as part of vaccination development attempts. Additionally, we should remember that cytokine profiles at a tissue level (e.g. in the spleen) were not assessed, and these could provide further insights into haemoplasma virulence.

To further our knowledge of haemoplasma virulence, comparative studies using *E. coccoides* and different strains of *M. haemomuris*, i.e. haemoplasmas of differing pathogencity, could be used. Such studies would require haemoplasma genome and encoded protein data to assess the roles of different haemoplasma proteins during infection. Whole genome sequences have only been derived for haemoplasmas grown in sizeable hosts that allowed comparably easy collection of larger blood volumes from which to attain enough haemoplasma DNA for whole genome sequencing. This could not be attempted with our mouse model due to the large number of infected mice that would be required. However, once putative haemoplasma virulence proteins are known, their role in infection in mice could be explored similarly to previous attempts in cats (85, 94) and pigs (93, 117) that used a combination of screening protein preparations, mass spectrometry and recombinant expression of putatively immunoreactive proteins but would benefit from already existing methods of investigating the mouse immune system, their low costs and the availability of inbred strains. However, such studies would still be limited by the small volumes of blood that can be collected in mice. An alternative approach to investigate haemoplasma pathogenicity would be to evaluate haemoplasma attachment to erythrocytes *in vitro* but this requires successful *in vitro* cultivation of *M. haemomuris*.

6.3 IN VITRO CULTIVATION OF *M. HAEMOMURIS*

We focused some of our studies (Chapter 5) on techniques to cultivate *M. haemomuris in vitro*. We used the mouse haemoplasma model to generate viable haemoplasmas and used sentinel mice as binary assays (i.e. infected versus uninfected) to detect *in vitro* maintenance and viability of haemoplasmas despite the absence of *in vitro* growth. Culture media compositions and additives were partially dictated by our objective to infect murine erythroblast precursors (e.g. StemPro™-34 serum-free medium, insulin, EPO) and partially based on genome analysis results to define the metabolic pathways present or absent (79). Previous *in vitro* culture experiments that had delivered promising results were also used to inform which additives to use e.g. hypoxanthine (216, 241) and transferrin (242).

We were able to maintain *M. haemomuris* viability for 8 days under *in vitro* conditions and support its *in vitro* growth for 4 consecutive days (*in vitro* cultivation studies 5 and 6, Chapter 5). A continuous *in vitro* cultivation amplification system could not be developed in the present study, so this does present a continued challenge in haemoplasma research. Such a system would allow for log-fold increases in *M. haemomuris* genome copies *in vitro* to generate haemoplasma numbers

large enough to be suitable for whole genome sequencing and proteomic studies. Future experiments could comprise the systematic removal of additives from media (e.g. insulin but also components included within the StemPro™-34 medium) to clearly define growth requirements before attempting to increase culture yields. Ideally, the use of mouse blood in cultivation studies could be avoided by adding substrates containing putative receptors and other growth requirements that are normally provided in blood. However, it is possible that *M. haemomuris* requires metabolically active erythrocytes to grow, and future research may need to focus on the development of erythroblast precursors to a suitable level of maturation to express putative receptors and provide metabolic susbstrates for the growth of *M. haemomuris*. The application of our *in vitro* cultivation system to other haemoplasma species, such as *M. haemofelis* or *M. suis*, could well be successful, but has not yet been attempted due to this needing a source of viable haemoplasmas from respective hosts (i.e. cats or pigs). However, sourcing such haemoplasmas experimentally poses ethical and economic problems unfortunately.

6.4 FUTURE WORK

Obtaining the whole genome sequence of our *M. haemomuris* isolate should be a priority of any future work with the mouse model and *in vitro* cultivation system presented within this thesis. Gene sequences would allow direct inference of metabolic needs for in *vitro* cultivation work and subsequently enable translatability of *in vitro* cultivation and any mouse model findings to other haemoplasma species. With a sufficiently developed *in vitro* cultivation system, e.g. comprising expontential haemoplasma expansion and the addition of recombinantly expressed host erythrocyte or host plasma components, haemoplasma proteins could be produced using the haemoplasmas themselves as an expression system. This would lead to the first ever accurate mass spectrometric analysis of a haemoplasma proteome, providing viable insights into all haemoplasma species as no such data is available to this date.

Since the whole genome sequence of the feline haemoplasma *M. haemofelis* is already available, an alternative to further developing a translatable *in vitro* cultivation system of *M. haemomuris* would comprise the *in vitro* cultivation of *M. haemofelis*, using red blood cells from cats diagnosed with polycythaemia vera (a neoplasm of the red blood cell lineage) which could replace the murine erythrocytes used in the current studies with *M. haemomuris*. As a very first step, the experiment presented in 5.2.2.7 would need to be repeated with the refined methodology of later experiments (gentle rocking and timing of blood collection) to answer the question as to whether *M. haemomuris* requires red blood cells alone or other plasma components to grow. If red blood cells are the only substrate requirement (apart from already added components and media) a clonal population from a polycythaemic cat could be used to rapidly source substrate for in vitro cultivation of *M. haemofelis*.

Once *in vitro* cultivation has led to genome sequencing and increased our knowledge of the *M. haemomuris* proteome, immunogenic *M. haemomuris* proteins can be assessed for their ability to establish a protective immune response. The knowledge of involved immunological pathways will hereby help keep mouse numbers at a minimum as cytokine measurements during infection can be used to further these experiments even in the absence of protection from infection.

Ultimately the work presented in this thesis will lead to *in vitro* cultivation of non-murine haemoplasmas, allowing generation of DNA for haemoplasma whole genome sequencing and proteins for mass spectrometry. These will inevitable lead to closer insights into host-pathogen interaction of veterinary relevant haemoplasmas and the development of vaccination or more targeted treatment strategies for clinical haemoplasmosis in cats and other veterinary species.

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8 APPENDIX A

ABBREVIATIONS

Abbreviations and acronyms used in the main text:

Abbreviation	Meaning
cDNA	Complementary DNA (derived from reverse transcription)
COG	Clusters of Orthologous Groups
IL-10	Interleukin-10
IL-1b	Interleukin-1b
LI	Lobularity Index
APC	Antigen Presenting Cells
AUC	Area Under the Curve
BLAST	Basic Local Alignment Search Tool
BHQ2	Black Hole Quencher 2
CHCM	Cell Haemoglobin Concentration Mean
CH	Cell Haemoglobin Content
CI	Confidence Interval
%CV	Coefficient of Variation
CRFK	Crandell Rees Feline Kidney (cell line)
CMP	Cytidine Monophosphate
DNA	Deoxyribonucleic Acid
Df	Degrees of Freedom
DMSO	Dimethyl Sulfoxide
DDH	DNA-DNA Hybridisation
dpi	Days Post Inoculation
DMEM	Dulbecco's Modified Eagle's Medium
RDW	Red Cell Distribution Width
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin (human recombinant)
F	F-Statistic
FAM	6-Carboxyfluorescein;
BHQ	Black Hole Quencher
FIV	Feline Immunodeficiency Virus
GCSF	Granulocyte Colony Stimulating Factor
gDNA	Genomic DNA
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
DNAK	Heat-Shock Protein 70
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
HCT	Haematocrit
Hb	Haemoglobin Concentration

HDW	Haemoglobin Concentration Distribution Width
HF	So-called ‘Haemofelis’ (HF)-Group of Haemoplasmas
HM	So-called ‘Haemominutum’ (HM)-Group of Haemoplasmas
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IL	Interleukin
IL-12p40	Interleukin-12 Subunit p40
IL-12p70	Interleukin-12 Subunit p70
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-1a	Interleukin-1a
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-9	Interleukin-9
IVC	Individually Ventilated Cage
ITS	Internally Transcribed Spacer (region)
KC	Keratinocyte Chemoattractant (CXCL1)
Li-heparin	Lithium-heparin
LoD	Limit of Detection
LoQ	Limit of Quantification
LUC	Large Unstained Cells
MIP1	Macrophage Inflammatory Protein 1
MWU	Mann-Whitney-U statistic
MCP1	Mast Cell Protease 1 (CCL2)
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MPC	Mean Platelet Component
MPM	Mean Platelet Mass
MPV	Mean Platelet Volume
mRNA	Messenger RNA
MFE	Minimum Free Energy
MIP1a	Macrophage Inflammatory Protein 1a
MIP1b	Macrophage Inflammatory Protein 1b
MSG1	GAPDH
MLSA	Multi-locus Sequence Analysis
MSA	Multiple Sequence Alignments
MPXI	Myeloperoxidase Index
NCBI	National Center for Biotechnology Information
NK)	Natural Killer Cells
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction

PBS	Phosphate-Buffered Saline
PLT	Platelet Count
PDW	Platelet Distribution Width
PCT	Plateletcrit
PP	Polypropylene
qPCR	Quantitative PCR
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted (cytokine)
RBC	Red Blood Cell Count
RT-qPCR	Reverse Transcriptase qPCR
RPMI	Roswell Park Memorial Institute (culture medium)
RNA	Ribonucleic Acid
SCF	Stem Cell Factor
SPF	Specified-Pathogen-Free
stdev	Standard Deviations
streptavidin-PE	Streptavidin-Phycoerythrin
TEX	Texas Red
Th	T-helper Cell
Ct	Threshold Cycle
Tm	Melting Temperature
TNF-α	Tumor Necrosis Factor Alpha
WBC	Total Nucleated Cell Count
Tregs	T-regulatory Cells
WBCP	White Blood Cell Count by Peroxidase

Nucleotide symbols as applied by the International Union of Applied Chemistry (IUPAC):

Nucleotide symbol	Nucleobase
G	Guanine
A	Adenine
T	Thymine
C	Cytosine
U	Uracil
R	G or A
Y	T or C
M	A or C
K	G or T
S	G or C
W	A or T
H	A or C or T
B	G or T or C
V	G or C or A
D	G or A or T
N	any nucleobase (G, A, T, C)

9 APPENDIX B

DNA SEQUENCES USED FOR INITIAL IDENTIFICATION OF NOVEL RODENT HAEMOPLASMA ISOLATES

Sequence Name	DNA Sequence
Isolate. #6	ATATATTCAAGAACACCAGAGGCGAAGGCGAAAACTTAGGCCGATATTGACGCTTAGGCTCGAAAGTGTGGGGA
Isolate #8	ATATATTCAAGAACACCAGAGGCGAAGGCGAAAACTTAGGCCGATATTGACGCTTAGGCTCGAAAGTGTGGGGA
Isolate #9	ATATATCCAAGAACACCAGAGGCGAAGGCGGAAACTTATACCGACATTGACGCTTAGGCTCGAAAGCGTGGGTA
Isolate #13	ATATATTCAAGAACACCAGAGGCGAAGGCGGAAACTTAGGCCGCTATTGACGCTTAGGCTTGAAAGTGTGGGGA
Isolate #18	ATATATTTAAGAACACCAGAGGCGAAGGCGAAAACTTAGGCCATTATTGACGCTTAGGCTTGAAAGTGTGGGTA

Table A-B1 *DNA sequences of amplicons of the partial 16S rRNA gene of the five rodent haemoplasma isolates as amplified from the generic haemoplasma qPCR assay.*

ISOLATE #6 (*M. HAEMOMURIS*) 16S RRNA GENE SEQUENCE

The near-complete 16S rRNA gene sequence, ITS sequence and partial 23S rRNA gene sequence for isolate #6 is given below:

ATACATGCAAGTCGAGCGGACCGCTAGCAATAGCGGTTAGCGGCGAACGGGTGAGTAATGAATACTTAACAT
ACCTCCATGAAGGAAATAGCTATTCGAAAGAGTAATTAATGTCCTATAGGAGCTAGCCTCACATGAGGTTGGC
TTTAAAGGCGCAAGCCACTTGGAGATTGGAGTATTTTCTATTAGCTAGTTGGCGGGATAACAGCCCACCAAGG
CAGTGATAGATAGCTGGTCTAAGAGGATGAACAGCCACAATGGGATTGAGATACGGCCCATATTCCTACGGG
AAGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGTGATGCCATGTGAACGATGAAGGTCT
TTTTGATTGTAAAGTTCTTTTATTGGGGAAAATGATGATGGTACCCAGTGAATAAGTGACAGCAAACTATGTG
CCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGA
TTGATAAGTTCTGTGTTAAATGCAGCCGCTCAACGGTTGTATGCGCAGAATACTGTTTTTCTAGAATACGGTAG
AAAGTTTTGGAATTGAATGTGGAGCGGTGGAATGTGTAGATATATTCAAGAACACCAGAGGCGAAGGCGAA
AACTTAGGCCGATATTGACGCTTAGGCTCGAAAGTGTGGGGAGCAAATGGGATTAGATACCCCAGTAGTCCA
CACCGTAAACGATGGATATTAGATGTTGGGACTTGAGTCTCAGCGTTGTAGCTTACGTGTTAAATATCCCGCCT
GAGTAGTACATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCTGAACAAGTGGTGGAACATGTTGCT
TAATTCGACCATACACGAAAAACCTTACCAAGATTTGACATCCCCTGCGAAGCTTTAGAAATAAAGTGGAGGT
TATCAGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCATGAGATGTCTGGTTAAGTCCTGAAACGA
GCGCACCCTACTCTTTAGTTAACTTTCTAAAGAGACTGAACAGTAATGTATAGGAAGGATGGGATCACGTCAA
GTCATCATGCCCCTTATATCTTGGGCCGCAAACGTGTTACAATGGTGGGTACAGCGTGTCGCAAGCCAGCGAT
GGCAAGCTAATCACTAAAAGCCCATCTCAGTCCGGATAAAAGGCTGCAATTCGCCTTTTTGAAGTTGGAATCA
CTAGTAATCCCGTGTCAGCTATATCGGGGTGAATACGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGA
GAGGGAGAGGCATTCGAAAACGTATTTGTTGCGTCTAGAATGAATTTCCTGATTGGAGTTAAGTCGTAACAAG
GTACCCGTACGAGAACGTGCGGGTGGATAATCTTTTGTATTCCGCTTTTAATTAGCGGAAGTGCATATAAACA
GAATGAGTTTTACTTAATGATGGACAACGAATAGGAGTATTGTCCTATACACATTAAGGCGGATGGCTTTCAG
CTTTGAGAGAACTATCTCTCTGGGTTGTTTTTTGACAAGACAAAAGATAATAACCGAGTTAACTTTAAATAAAG
TTGAATTCATACGTTGAATAAGTTAAGAGCTAGAGGTGGATTTCTTGGAAATGGAAGGCGATGAAGGACGTG
TCAATCTGCGATAAGCCAGGGGAAGCTGATAAGAAGCCCCGATCCCTGGATTTCCGAATGTAGAAATACGAT
GTACCTCCGGTATATCATTTTGGAGTGAATACATAGCTCCAAAAAGCGAACCTCGTGAAGTGAAACATCTCAG
TAACGAGAGGAAAAGAAAACGAAGCGATTCCCTAAGTAGTGGTGAGCGAAAGGGGATTAGGACAAACCAGA
GCTTGCTCTGGGGTTGTAGGACTTCAACGTGGACTCTAGGGATATAGAAGAAATCTTTGGAACAAGATGGCA
TAGAGGGCGACCCCCCCGTATTCGAAATGTCCCTAGTACCTAGAAGTATCCTGAGTAGGGCGGGACACGTGT
AATCCTGT

ISOLATE #18 (*E. COCCOIDES*) 16S RRNA GENE SEQUENCE

The near-complete 16S rRNA gene sequence for isolate #18 is given below:

CGGATATATCCGCAAGGATATGTTAGTGGCGAACGGGTGAGTAATACATATTTAACATACCCCTTAGAGGGA
AATAGCCGCTTGAAAAAGCGATTAATGTCCCATAGGAACCCTTTCACAGGAAGGGAGTTTTAAAGGAGAAAT
CCGCTTTGGGATTGGAATATGCTCTATTAGTTAGTTGGCGGGGTAAAGGCCCACCAAGACTATGATAGATAGC
TGGTCTTAGAGGACGAACAGCCACAATGGGATTGAGATACGGCCCATATTCCTACGGGAAGCAGCAGTAGGG
AATCTTCCACAATGGACGAAAGTCTGATGGAGYRATGCCATGTGAACGATGAAGGTCATTTTGATTGTAAAGT
TCTTTTAGGGGGGAAAATAATGATGGTACCTCCTGAATAAGTGACAGCAAACTATGTGCCAGCAGCTGCGGT
AATACATAGGTCGCAAGCGTTATTCGGATTTATTGGGCGTAAAGCAAGCGCAGGCGGATGAATAAGTTCTGT
GTTAAAAGCAGCTGCTCAACAGTTGTTTGCACCGAATACTATTCGTCTAGAATGTGGTAGGAAGTTTTGGAAT
TAAATATGGAGCGGTGGAATGTGTAGATATATTTAAGAACACCAGAGGCGAAGGCGAAAACTTAGGCCATTA
TTGACGCTTAGGCTTGAAAGTGTGGGTAGCAAATGGGATTAGATACCCCAGTAGTCCACACCGTAAACGATG
GGTATTAGGTGCCGGGGTTAGAGCTTCGGTGCTGTAGCTTACGTGTTAAATACCCCGCCTGGGTAGTACATAT
GCAAATATGAAACTCAAAGGAATTGACGGGGACCTGAACAAGTGGTGGAACATGTTGCTTAATTCGATAATA
CACGAAGAACCTTACCAAGGTTTGACATCCCTCGCRAAACCATAGAAATATGGCGGAGGTTATCAGGGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCATGAGATGTTTGGTTAAGTCCCGCAACGAGCGCAACCCTACT
CTTTAGTTAATTTATCTAAAGAGACTGAACAGTAATGTATAGGAAGGATGGGATCACGTCAAATCATCATGCC
CCTTATGCCTTGGGCTGCAAACGTGTTACAATGGGGAGTACAATGTGTCGCAAACTAGCGATAGTGAGCTAAT
CACCAAAGCTTCCCTCAGTTCGGATAAAAGGCTGCAATTCGCCTTTTTGAAGTTGGAATCACTAGTAATCCCGT
GTCAGCCATATCGGGGTGAATACGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGAGAGGGAGGGGC
ATTTAAAAACATATTGATTATT

ISOLATE #13 (*M. HAEMOMURIS*-LIKE HAEMOPLASMA) 16S RRNA GENE SEQUENCE

The near-complete 16S rRNA gene sequence, ITS sequence and partial 23S rRNA gene sequence for isolate #13 is given below:

ACAATGGGCGAAAGCCTGATGGAGCGATGCCATGTGAATGATGAAGGTCTTTTTGATTGTAAAGTTCTTTTAT
TGGGGAAAATGATGATGGTACCCGATGAATAAGTGACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATA
GGTCGCGAGCGTTATTCGGATTTATTGGGCGTAAAGCAAGCGCAGGCGGACGAGTAAGTTCTGTGTTAAAAA
CAGCAGCTCAACTGTTGTTTGCGCCGAATACTGCTCGTCTAGAATACGGTAGAAAGTTTCGGAATTGAATATG
GAGCGGTGGAATGTGTAGATATATTCAAGAACACCAGAGGCGAAGGCGGAAACTTAGGCCGCTATTGACGCT
TAGGCTTGAAAGTGTGGGGAGCAAATGGGATTAGATACCCCAGTAGTCCACACCGTAAACGATGGATATTAG
ATGTTGGGACTTGAGTCTCAGCGTTGTAGCTTACGTGTTAAATATCCCGCCTGAGTAGTACATATGCAAATATG
AAACTCAAAGGAATTGACGGGGACCTGAACAAGTGGTGGAACATGTTGCTTAATTCGACCATACACGAAAAA
CCTTACCAAGATTTGACATCCCCCGCGAAACTCTAGAAATAGAGCGGAGGTTATCGGGGTGACAGGTGGTGC
ATGGCTGTCGTCAGCTCGTGTCATGAGATGTTTGGTTAAGTCCCGCAACGAGCGCAACCCTACTCTTTAGTTAA
TTTTTTAAAGAGACTGCACAGTAATGTAGAGGAAGGATGGGATCACGTCAAGTCATCATGCCCCTTATATCTT
GGGCCGCAAACGTGTTACAATGGCGAGTACAGCGTGTCGCAAACCAGCGATGGTAAGCTAATCACTAAAAGC
TCGTCTCAGTCCGGATAAAAGGCTGCAATTCGCCTTTTTGAAGTTGGAATCACTAGTAATCCCGTGTCAGCCAT
ATCGGGGTGAATACGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGAGAGGGAGAGGCATTCRAAAA
TCGAATAATTAAGTCGTAACAAGGTACCCGTACGAGAACGTGCGGGTGGATAATATTCTTTGAAGTTTATTTA
CTACATAAACTAAATAAAAAATGAATGAGGTTAACTAGATGATAACTGTTGCTAGAGTCTTCAGTTTGTAACAT
CTAGGCGGATGACTTTCAGCTTTGAGAGAACTATCTAATGATTCTCTCAGAGATTGTTTTTTGAAATTGAAAAG
ATAATAACCGAGTTAACTTGATAAAAGTTAAATTCATGCGTTGAATAAACTAAGAGCTAGAGGTGGATTCCTT
GGAAATGGAAGGCTATGAAGGACGTGCTAATCTGCGATAAGCCTGGGGGAGTTGATAAGAAACTGTGATCC

CTGGATCTCCGAATGTAGAAATACGATAGACCATTAGGTCCATCATTTTGAGGCTAATAAATAATCTCAAAAA
GCGAACCTCGTGAAGTGAAACATCTCAGTAACGAGAGGAAAAGAAAACGAATGTGATTCCCTAAGTAGTGGT
GAGCGAAAGGGGATTAGGACAAACCGGATTCGTCCGGGGTTGTAGGACGTCAATGTGGACTTTGAGAGTAT
AGAAGAAATATTTGGAACAATATGGCAAAGAGGGCGAACCCCCCCGTATTCGAAATATTCTCAATACCTA

For comparison, the corresponding 16S rRNA sequence of the previously unpublished 'vole haemoplasma' is given below:

CTGATGGCATGCATAATACATGCAAGTCGAGCGAACTGCTAGCAATAGCAGTTAGCGGCGAACGGGTGAGTA
ATGCATACTTAACATGCCTCTCCGAGGGGAATAGCTACCCGAAAGGGTAATTAATACCCCATACTAGCCCCCTC
ACATGAGGGTGGCTTTAAAGGAGCAATCCGCGGGGAGATTGGAGTATGTCCCATTAGCTTGTTGGCGGGGTA
ATGGCCCACCAAGGCAATGATGGGTAGCTGGTCTAAGAGGATGAACAGCCACAATGGGATTGAGATACGGC
CCATATTCCTACGGGAAGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCGATGCCATGT
GAATGATGAAGGTCTTTTTGATTGTAAAGTTCTTTTATTGGGGAAAATGATGATGGTACCCGATGAATAAGTG
ACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCAAGCGTTATTCGGATTTATTGGGCGTAAAG
CAAGCGCAGGCGGACGAGTAAGTTCTGTGTTAAAAACAGCAGCTCAACTGTTGTTTGCGCCGAATACTGCTCG
TCTAGAATACGGTAGAAAGTTTCGGAATTGAATATGGAGCGGTGGAATGTGTAGATATATTCAAGAACACCA
GAGGCGAAGGCGGAAACTTAGGCCGCTATTGACGCTTAGGCTTGAAAGTGTGGGGAGCAAATGGGATTAGA
TACCCCAGTAGTCCACACCGTAAACGTTGGATATTAGATGTTGGAACTTGAGTTTCAGCGTTGTAGCTTACGTG
TTAAATATCCCGCCTGAGTAGTACATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCTGAACAAGTG
GTGGAACATGTTGCTTAATTCGACAATACACGAAAAACCTTACCAAGATTTGACATCCCTTGCAAAACCCTAGA
GATAGGGCGGAGGTTATCAGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCATGAGATGTTTGGTT
AAGTCCCGCAACGAGCGCAACCCTACTCTCTAGTTAGTTGTCTAGAGAGACTGCACAGTAATGTAGAGGAAG
GATGGGATCACGTCAAGTCATCATGCCCCTTATATCTTGGGCCGCAAACGTGTTACAATGGCGAGTACAGCGT
GTCGCAAACCAGCGATGGTAAGCTAATCACTAAAAGCTCGTCTCAGTCCGGATAAAAGGCTGCAATTCGCCTT
TTTGAAGTTGGAATCACTAGTAATCCCGTGTCAGCCATATCGGGGTGAATACGTTCCCAGGTCTTGTACACACC
GCCCGTCAAACTATGAGAGGGAGAGGCATTCAAAAACGAATTAATTTACGTCTAGAGTGAATTTCCTGATTGG
AG

ISOLATE #9 (NOVEL RODENT HAEMOPLASMA) 16S RRNA GENE SEQUENCE

The partial 16S rRNA gene sequence for isolate #9 is given below:

TGAACGACGAAGGTCTTATTGATTGTAAAGTTCTTTTAGAGGAAAAATACTGATGGTACCTCTTGAATAAGTG
ACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGATTTATTGGGCGTAAA
GCGAGCGCAGGCGGATGGGTAAGTTCTGGTGTTAAAAATAGCCGCTCAACGGTTATTTGCGCCGAATACTGC
TCATCTTGAATGCGGCAGAAAGTTTCGGAATTGGATGTGGAGCGGTGGAATGTGTAGATATATCCAAGAACA
CCAGAGGCGAAGGCGGAAACTTATACCGACATTGACGCTTAGGCTCGAAAGCGTGGGTAGCAAATGGGATT
AGATACCCCAGTAGTCCACGCCGTAAACGATGGATATTAGATGTTGGGGACGTGATCCTCAGCGTTGTAGCTT
ACGTGTTAAATATCCCGCCTGGGTAGTACATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCTGAAC
AAGTGGTGGAACA

ISOLATE #8 (*M. HAEMOMURIS*) 16S RRNA GENE SEQUENCE

The partial 16S rRNA gene sequence for isolate #8 is given below:

TGAACGATGAAGGTCTTTTTGATTGTAAAGTTCTTTTATTGGGGAAAATGATGATGGTACCCAGTGAATAAGT
GACAGCAAACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGATTTATTGGGCGTAA
AGCGAGCGCAGGCGGATTGATAAGTTCTGTGTTAAATGCAGCCGCTCAACGGTTGTATGCGCAGAATACTGTT
TTTCTAGAATACGGTAGAAAGTTTTGGAATTGAATGTGGAGCGGTGGAATGTGTAGATATATTCAAGAACACC

AGAGGCGAAGGCGAAAACTTAGGCCGATATTGACGCTTAGGCTCGAAAGTGTGGGGAGCAAATGGGATTAG
ATACCCCAGTAGTCCACACCGTAAACGATGGATATTAGATGTTGGGACTTGAGTCTCAGCGTTGTAGCTTACG
TGTTAAATATCCCGCCTGAGTAGTACATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCTGAACAAGT
GGTGGAACA

PHYLOGENY OF ALL NOVEL RODENT HAEMOPLASMAS PRESENTED WITHIN THIS STUDY

A phylogenetic tree of all five novel haemoplasma isolates was constructed using a partial, 463bp 16S rRNA gene sequence and is presented in **Figure A-B1**.

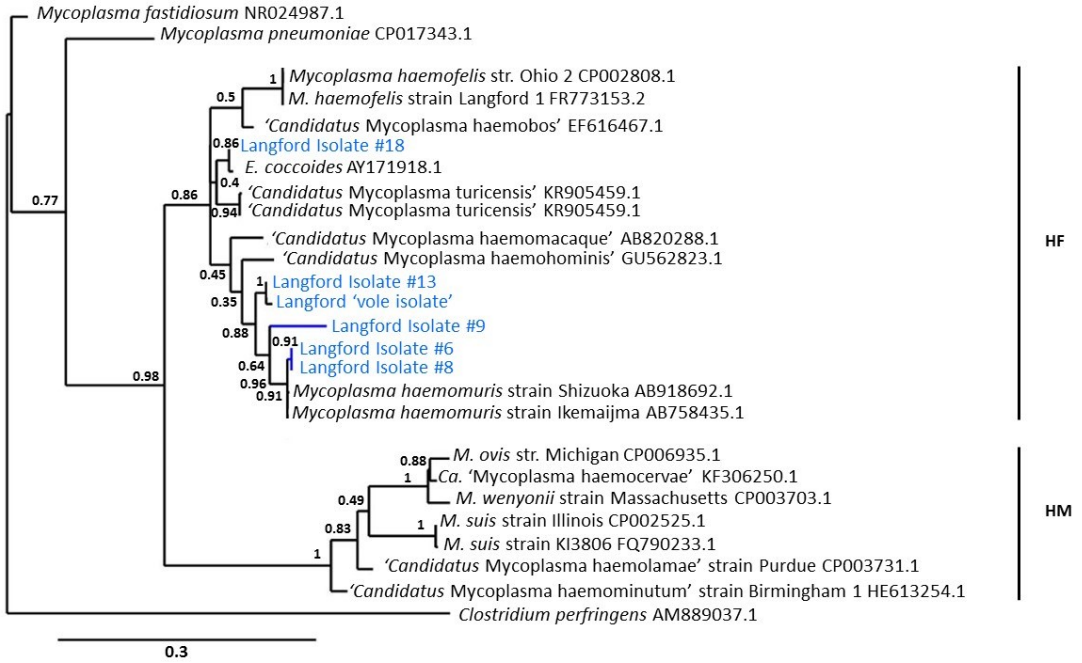

Figure A-B1 *Maximum likelihood phylogenetic tree of 16S rRNA gene sequences based on a 463 bp sequence that was analysed for haemoplasmas and other Mycoplasma species. The dataset was sampled 1000 times and bootstrap values are given as percentages where nodes occur. HF, haemofelis subgroup of haemoplasmas; HM, haemominutum subgroup of haemoplasmas. Isolates derived from this study are highlighted in blue. Branches comprising bootstrap values of less than 0.5 have been included for illustrative reasons.*

10 APPENDIX C

Additional data and interpretations of the PCA carried out as part of the cytokine expression study will be presented within this appendix.

SCREE PLOT

A Scree plot, listing eigenvalues of the extracted components was constructed to allow visual assessment of 'breaks' in eigenvalue to limit the number of meaningful components and is presented in **Figure A-C1**.

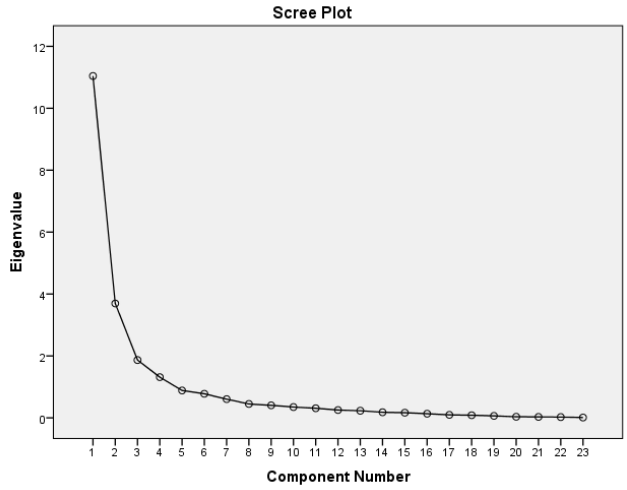

Figure A-C1 *Scree plot of extracted components. Eigenvalues are plotted on the y-axis and their respective components on the x-axis. Components 1, 2, 3 and 4 were selected based on eigenvalues > 1 and no further observed 'breaks' (sudden decreases) in eigenvalues along the plot.*

UNROTATED PCA SOLUTION DERIVED FROM % CHANGE

Component loadings, derived from percentage of change from baseline (represented by values obtained on day 0 of the experiment) are presented in **Table A-C1**. The component loadings did not exhibit a different pattern or change the interpretation of extracted components when compared to component loadings extracted from absolute cytokine values and were hence not explored further.

Cytokine	Component			
	1	2	3	4
IL1a %	**0.803**	-0.012	0.305	0.092
IL1b %	**0.825**	-0.446	0.076	0.004
IL2 %	0.263	**0.674**	0.437	-0.184
IL3 %	**0.912**	-0.128	-0.079	0.309
IL4 %	**0.894**	0.153	0.072	0.082
IL5 %	**0.832**	-0.271	-0.082	0.028
IL6 %	**0.723**	-0.027	-0.183	0.418
IL9 %	**0.918**	-0.064	0.139	-0.075
IL10 %	**0.614**	0.301	-0.273	-0.477
IL12p40 %	**0.550**	0.481	-0.343	-0.141
IL12p70 %	**0.832**	-0.138	0.241	-0.057
IL13 %	**0.691**	-0.304	0.466	-0.079
IL17 %	0.057	**0.756**	0.265	0.208
Eotaxin %	-0.429	0.298	0.395	0.420
GCSF %	**0.583**	0.308	-0.423	0.170
GMCSF %	**0.816**	-0.112	-0.249	0.032
IFNgamma %	**0.915**	0.088	0.204	-0.007
KC %	**0.514**	-0.166	**0.609**	-0.322
MCP1 %	**0.763**	0.024	0.054	-0.157
MIP1a %	**0.708**	0.361	-0.306	-0.399
MIP1b %	**0.854**	0.256	-0.212	0.152
RANTES %	-0.155	**0.869**	0.179	0.082
TNFalpha %	**0.878**	0.003	-0.022	0.379

Table A-C1 *Component matrix as extracted by PCA for components with eigenvalues > 1 when using % change instead of absolute values (pg/ml). Factor loadings represent the correlation between the original variables and the extracted component and were considered significant for their respective components if they exhibited a loading greater than 0.5 for that component and less than 0.5 for all other components. These are indicated by shaded cells and bolded text. Cumulatively explained variance amounted to 77.413 %, comprising 50.074 % through factor 1, 13.380 % through factor 2, 8.528 % through factor 3 and 5. 431% through factor 4). This PCA was not significantly different to the solution derived from absolute values so was not further explored.*

MULTIVARIATE ASSESSMENT OF CHRONOLOGICAL CHANGES IN CYTOKINE EXPRESSION

Unrotated component scores were calculated and visually assessed for distribution of errors and homogeneity of variance as before. Results of univariate analyses on the first four unrotated component scores (assigning each individual cytokine value a score on a given component) and experimental groups (re-inoculation group versus naïve group), time (dpi) and interactions between the experimental group and dpi are presented in **Table A-C2**. In contrast to **Table 19**, the time (dpi) variable was entered as a covariate (rather than a fixed factor), hence forcing the analysis to assess significant changes over time and not only within the entire dataset.

Dependent variable:		**Component scores on principal component 1**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	18.256	3	6.085	8.421	**0.000**
Intercept	3.012	1	3.012	4.168	0.046
Group	2.225	1	2.225	3.080	0.085
Dpi	6.709	1	6.709	9.284	**0.004**
Group * Dpi	1.212	1	1.212	1.678	0.201
Error	39.744	55	0.723		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .315 (Adjusted R Squared = .277)					
Dependent variable:		**Component scores on principal component 2**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	12.909	3	4.303	5.249	**0.003**
Intercept	0.034	1	0.034	0.041	0.841
Group	3.711	1	3.711	4.526	**0.038**
Dpi	0.006	1	0.006	0.007	0.932
Group * Dpi	0.542	1	0.542	0.661	0.420
Error	45.091	55	0.820		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .223 (Adjusted R Squared = .180)					
Dependent variable:		**Component scores on principal component 3**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	8.089	3	2.696	2.971	**0.040**
Intercept	0.100	1	0.100	0.111	0.741
Group	1.908	1	1.908	2.103	**0.153**
Dpi	0.246	1	0.246	0.271	**0.605**
Group * Dpi	7.320	1	7.320	8.066	**0.006**
Error	49.911	55	0.907		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .139 (Adjusted R Squared = .093)					
Dependent variable:		**Component scores on principal component 4**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	.853	3	0.284	0.274	0.844
Intercept	0.432	1	0.432	0.416	0.522
Group	0.001	1	0.001	0.001	0.970
Dpi	0.832	1	0.832	0.801	0.375
Group * Dpi	0.003	1	0.003	0.002	0.961
Error	57.147	55	1.039		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .015 (Adjusted R Squared = .039)					

Table A-C2 *Univariate analyses of component scores (dependent variable) and experimental groups (naïve group versus re-inoculation group) and dpi (dpi in Phase 2 of the cytokine expression study). Significance was defined as p<0.05 and significant results are indicated by shaded cells and bolded text, results that became significant due to the interaction effect are indicated by bolded text alone. Note, that component scores reflect cytokine concentrations but cannot be interpreted as such. Group, experimental group (naïve group versus re-inoculated group); df, degrees of freedom; F, F-statistic; Dpi, days post inoculation.*

Another analysis, allowing for quadratic interactions of the time variable (indicated as 'Dpisqu') is presented in **Table A-C3**. Overall, no improved representation of the dataset was achieved by those additional analyses.

Dependent variable:		**Component scores on principal component 1**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	19.858	5	3.972	5.519	**0.000**
Intercept	0.011	1	0.011	0.016	0.900
Group	1.039	1	1.039	1.444	0.235
Dpi	5.936	1	5.936	8.249	**0.006**
Dpisqu	1.600	1	1.600	2.223	0.142
Group * Dpi	1.025	1	1.025	1.424	0.238
Group * Dpisqu	0.003	1	0.003	0.004	0.949
Error	38.142	53	0.720		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .342 (Adjusted R Squared = .280)					
Dependent variable:		**Component scores on principal component 2**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	14.441	5	2.888	3.514	0.008
Intercept	0.425	1	0.425	0.518	0.475
Group	2.758	1	2.758	3.356	0.073
Dpi	7.677E-05	1	7.677E-05	0.000	0.992
Dpisqu	1.031	1	1.031	1.255	0.268
Group * Dpi	0.527	1	0.527	0.641	0.427
Group * Dpisqu	0.391	1	0.391	0.475	0.494
Error	43.559	53	0.822		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .249 (Adjusted R Squared = .178)					
Dependent variable:		**Component scores on principal component 3**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	18.322	5	3.664	4.895	**0.001**
Intercept	6.715	1	6.715	8.969	0.004
Group	0.325	1	0.325	0.435	**0.513**
Dpi	0.614	1	0.614	0.820	**0.369**
Dpisqu	9.656	1	9.656	12.899	**0.001**
Group * Dpi	7.926	1	7.926	10.587	**0.002**
Group * Dpisqu	0.263	1	0.263	0.351	0.556
Error	39.678	53	0.749		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .316 (Adjusted R Squared = .251)					
Dependent variable:		**Component scores on principal component 4**			
Source	Type III sum of squares	Df	Mean square	F	Significance
Corrected model	9.503	5	1.901	2.077	0.083
Intercept	0.053	1	0.053	0.058	0.811
Group	5.050	1	5.050	5.519	0.023
Dpi	1.079	1	1.079	1.179	0.282
Dpisqu	0.127	1	0.127	0.138	0.711
Group * Dpi	0.052	1	0.052	0.056	0.813
Group * Dpisqu	8.309	1	8.309	9.081	0.004
Error	48.497	53	0.915		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .164 (Adjusted R Squared = .085)					

Table A-C3 *Univariate analyses of component scores (dependent variable) and experimental groups (naïve group versus re-inoculation group) and dpi (dpi in Phase 2 of the cytokine expression study). Significance was defined as p<0.05 and significant results are indicated by shaded cells and bolded text, results that became significant due to the interaction effect are indicated by bolded text alone. Note, that component scores reflect cytokine concentrations but cannot be interpreted as such. Group, experimental group (naïve group versus re-inoculated group); df, degrees of freedom; F, F-statistic; Dpi, days post inoculation.*

FACTOR ROTATION AND PATTERN MATRICES (FROM ABSOLUTE VALUES)

Solutions for the rotated PCA have been summarised in XXX and are presented below. **Table A-C4** lists the revised pattern matrix following promax rotation.

Cytokine	Rotated component			
	1	2	3	4
IL1a	.458	.334	-.148	**.593**
IL1b	**.515**	.467	-.069	-.344
IL2	-.152	.374	.139	**.852**
IL3	**1.002**	.014	-.090	-.110
IL4	**.684**	.274	.058	.169
IL5	**.583**	.221	.104	-.312
IL6	**1.101**	-.205	-.214	-.062
IL9	.497	**.516**	.070	-.024
IL10	-.283	.040	**1.048**	-.017
IL12p40	.126	-.274	**.755**	.263
IL12p70	.280	**.528**	.189	.050
IL13	.022	**.767**	-.142	-.063
IL17	-.019	-.103	.063	**.872**
Eotaxin	.019	-.062	-.575	**.558**
GCSF	**.710**	-.486	.313	.100
GMCSF	**.717**	.110	.129	-.225
IFNgamma	**.522**	.471	.083	.192
KC	-.321	**1.021**	.009	.150
MCP1	.034	.297	**.573**	.042
MIP1a	.070	.090	**.849**	-.023
MIP1b	**.691**	-.221	.429	.145
RANTES	-.011	-.089	.039	**.882**
TNFalpha	**1.056**	-.073	-.105	.098

Component correlation matrix

Component	1	2	3	4
1	1.000	.616	.641	-.042
2	.616	1.000	.396	-.151
3	.641	.396	1.000	.132
4	-.042	-.151	.132	1.000

Table A-C4 *Revised pattern matrix (Promax rotation with Kaiser normalisation) for components with eigenvalues > 1. Factor loadings considered significant are indicated by shaded cells and bolded text.*

It must be noted that the corresponding components (rotated component 1 to 4) of the rotated solution, although representing the same variance within the datapoints, do not correspond exactly with the unrotated component loading patterns. Non-orthogonal rotation was used which tries to optimise loadings by placing the axes in a way that maximises observed variance, even if factors are correlated and would show up more closely without or in an orthogonal rotation. As a result, individual cytokines may load on different components following rotation. Overall, no increase in interpretability was observed following rotation.

Visualisation of rotated component loadings after non-orthogonal (Promax) rotation

Rotated component scores are visualised in **Figure A-C2** (plotting rotated component scores over time) and **Figure A-C3** (scatterplot matrix of rotated component scores.

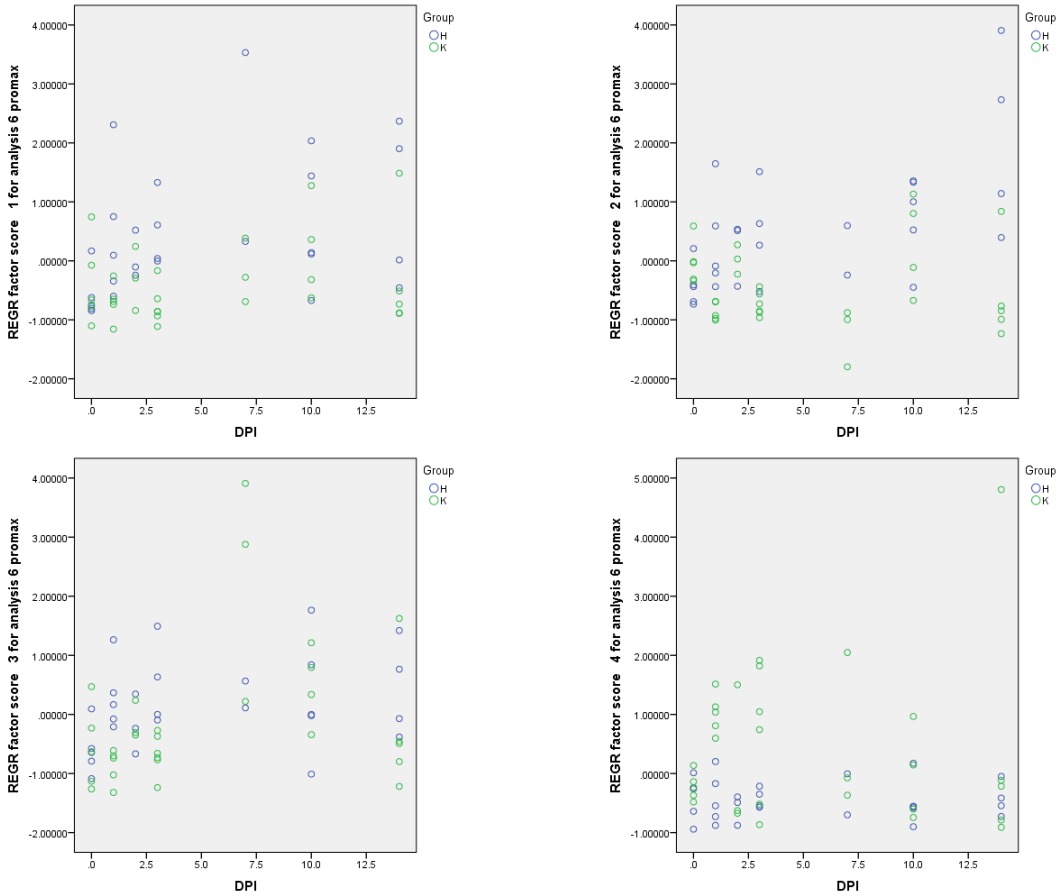

Figure A-C2 *Rotated component scores over time. Extracted component scores for each of the four rotated (Promax with Kaiser normalisation) components are plotted on the y-axis and days post inoculation (DPI) are plotted on the x-axis. Green: naïve group (K), blue: re-infected group (H).*

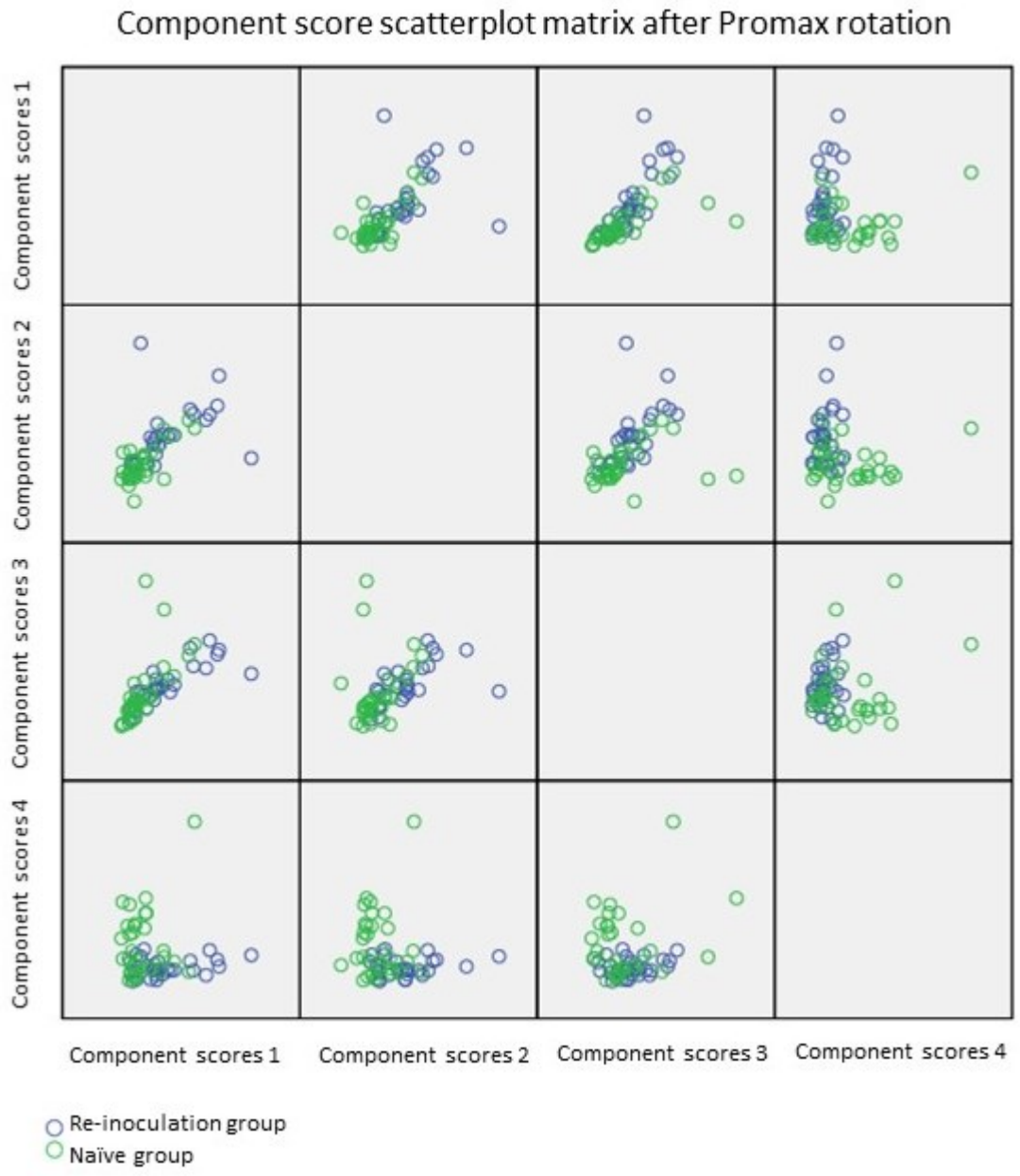

Figure A-C3 *Scatterplot matrix of unrotated component scores plotted against each other illustrating the relationships between the extracted components and clustering of the underlying dataset. Green circles represent component scores of the naïve group and blue circles represent component scores for the re-inoculation group. Component scores 1, component scores on rotated principal component 1; component scores 2, component scores on rotated principal component 2; component scores 3, component scores on rotated principal component 3; component scores 4, component scores 4 on rotated principal component 4; Dpi, days post inoculation.*

Univariate analyses after non-orthogonal (Promax) rotation

Rotated component scores were subject to univariate analyses as presented for the unrotated solution (**4.3.4.2.3**) and results are presented in **Table A-C5**.

Dependent variable:	**Component scores on rotated principal component 1**				
Source	Type III sum of squares	df	Mean square	F	Significance
Corrected model	24.173	13	1.859	2.474	**0.012**
Intercept	0.591	1	0.591	0.786	0.380
Group	11.258	1	11.258	14.977	**0.000**
Dpi	8.472	6	1.412	1.878	0.105
Group * dpi	6.421	6	1.070	1.424	0.227
Error	33.827	45	0.752		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .417 (Adjusted R Squared = .248)					
Dependent variable:	**Component scores on rotated principal component 2**				
Source	Type III sum of squares	df	Mean square	F	Significance
Corrected model	35.497	13	2.731	5.461	**0.000**
Intercept	0.048	1	0.048	0.097	0.757
Group	12.289	1	12.289	24.576	**0.000**
Dpi	9.688	6	1.615	3.229	**0.010**
Group * dpi	12.808	6	2.135	4.269	**0.002**
Error	22.503	45	0.500		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .612 (Adjusted R Squared = .500)					
Dependent variable:	**Component scores on rotated principal component 3**				
Source	Type III sum of squares	df	Mean square	F	Significance
Corrected model	30.772	13	2.367	3.912	**0.000**
Intercept	0.565	1	0.565	0.934	0.339
Group	0.172	1	0.172	0.284	**0.597**
Dpi	14.517	6	2.419	3.999	**0.003**
Group * dpi	11.487	6	1.915	3.164	**0.011**
Error	27.228	45	0.605		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .531 (Adjusted R Squared = .395)					
Dependent variable:	**Component scores on rotated principal component 4**				
Source	Type III sum of squares	df	Mean square	F	Significance
Corrected model	10.610	3	3.537	4.105	**0.011**
Intercept	0.017	1	0.017	0.020	0.887
Group	5.042	1	5.042	5.852	**0.019**
Dpi	0.001	1	0.001	0.001	0.981
Group * dpi	0.002	1	0.002	0.002	0.966
Error	47.390	55	0.862		
Total	58.000	59			
Corrected total	58.000	58			
R Squared = .183 (Adjusted R Squared = .138)					

Table A-C5 *Univariate analyses of rotated component scores (dependent variable) and experimental groups (naïve vs. re-infected) and dpi (days post inoculation/re-inoculation). Rotated scores allow for correlations between the scores but no not correspond directly to the unrotated solution. Significant results are highlighted in bold. Note, that regression factor scores reflect cytokine concentrations but cannot be interpreted as such. Rotation did not greatly help interpretation so was not further explored for analysis.*

Interpretation of Promax rotated PCA component loadings

Rotation is employed to increase interpretability. In the current case a non-orthogonal rotation matrix was explored to allow for correlation between individual cytokines and plot the intrinsic

variation of the dataset on coordinates, optimised for their differentiation. However, factors did not appear to allow further differentiation of immunological pathways, in fact, the Th17 pathway did not come up with significant effects on groups or days post inoculation after the rotation operation was executed. One may speculate that these non-orthogonal factors were more suitable to detect distinct peak-and-through patterns within the cytokine values over time, however, immunological interpretation was limited and hence, not further pursued.

CYTOKINE DECREASE/INCREASE OVER TIME (% CHANGE)

Individual cytokine concentrations of the naïve and re-inoculated group mice, exsanguinated at 0, 1, 2, 3, 7, 10 and 14 dpi as % change from baseline in are presented in **Figure A-C5.**

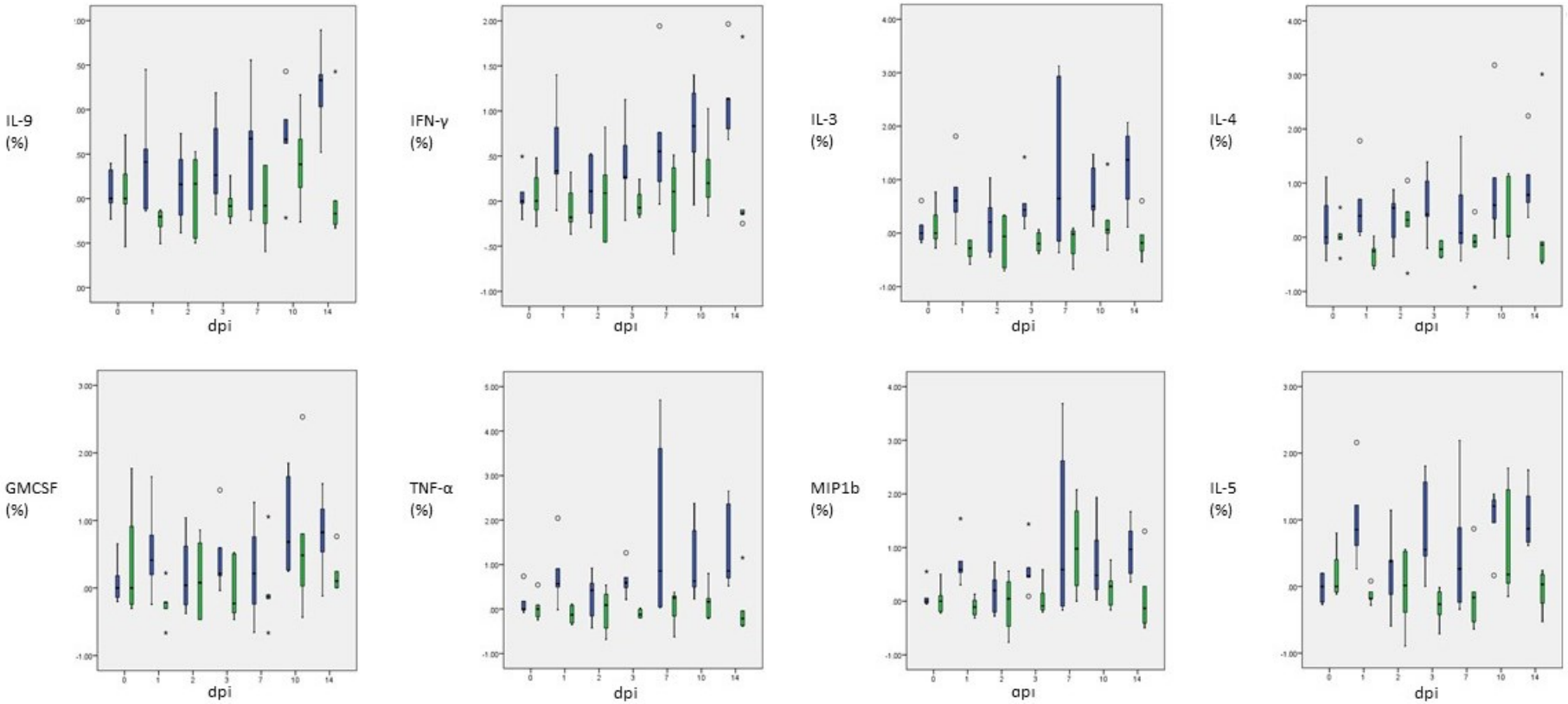

Figure A-C5*: Change in cytokine concentration over time for cytokines loading on principal component 1. A baseline value was defined as the median value of group 0 for each respective cytokine. Change over time was then calculated by subtracting the baseline from individually measured cytokines and dividing them by the baseline value. Cytokine concentrations (change from baseline, 1 = +100%) are plotted on the y-axis and days post inoculation are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated. Green: naïve group, blue: re-infected group.*

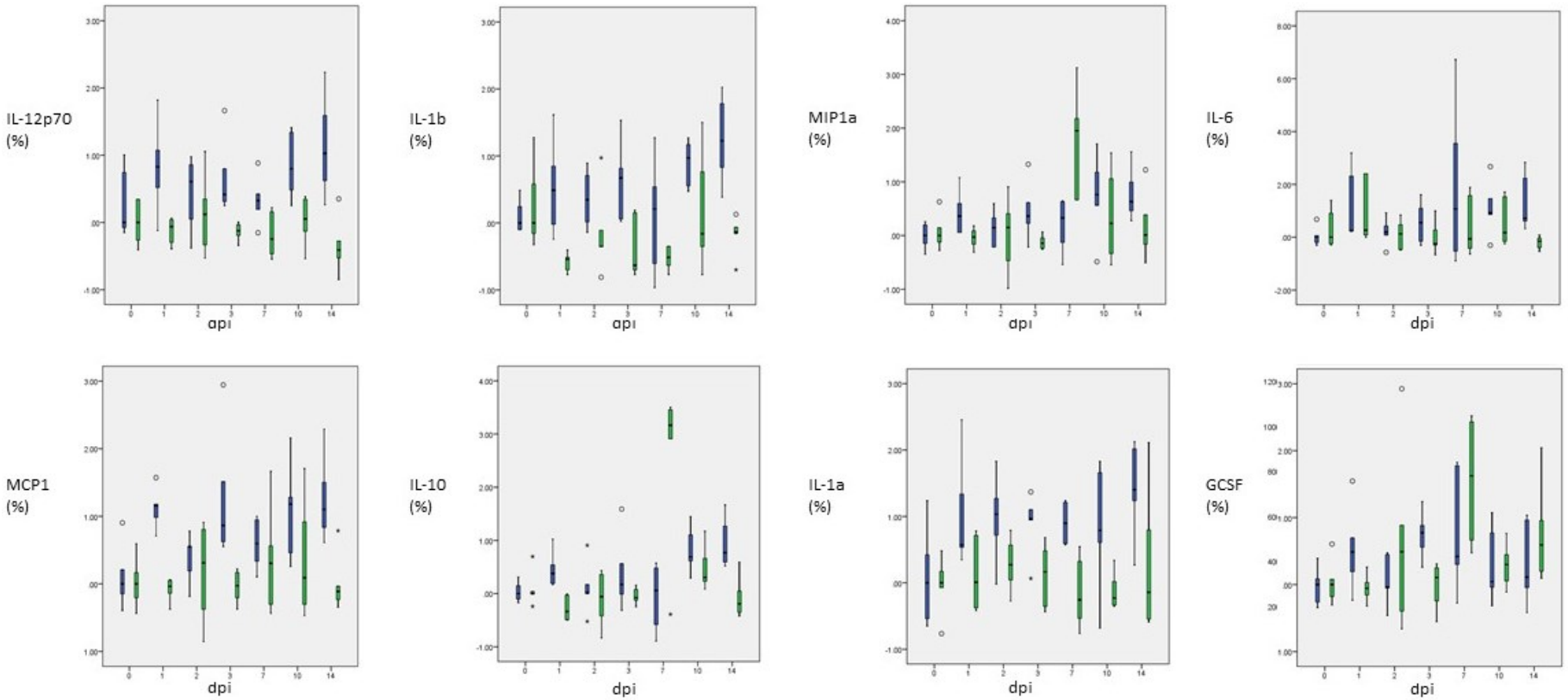

***Figure A-C5 (continues)**: Change in cytokine concentration over time for cytokines loading on principal component 1. A baseline value was defined as the median value of group 0 for each respective cytokine. Change over time was then calculated by subtracting the baseline from individually measured cytokines and dividing them by the baseline value. Cytokine concentrations (change from baseline, 1 = +100%) are plotted on the y-axis and days post inoculation are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated. Green: naïve group, blue: re-infected group.*

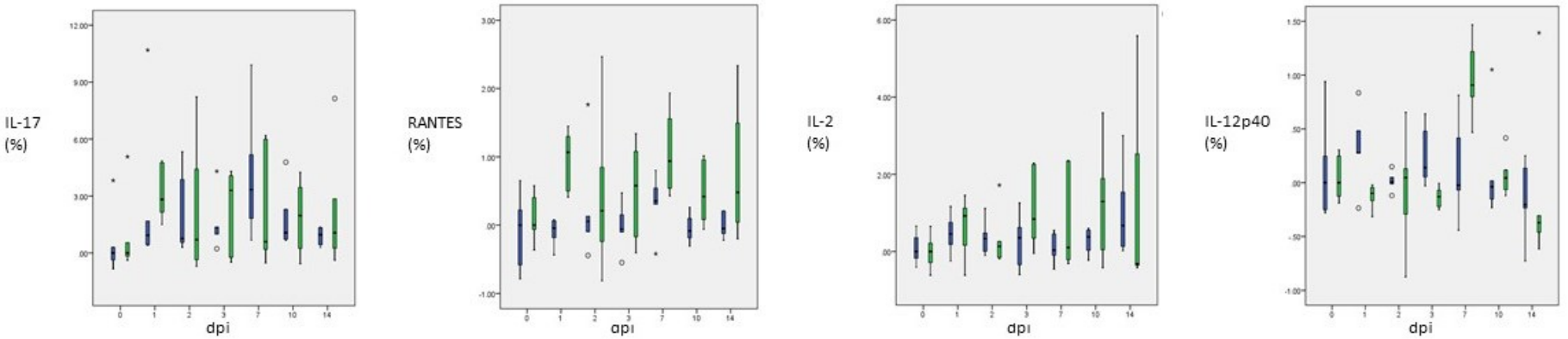

***Figure A-C5 (continued)**: Change in cytokine concentration over time for cytokines loading on principal component 2. A baseline value was defined as the median value of group 0 for each respective cytokine. Change over time was then calculated by subtracting the baseline from individually measured cytokines and dividing them by the baseline value. Cytokine concentrations (change from baseline, 1 = +100%) are plotted on the y-axis and days post inoculation are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated. Green: naïve group, blue: re-infected group.*

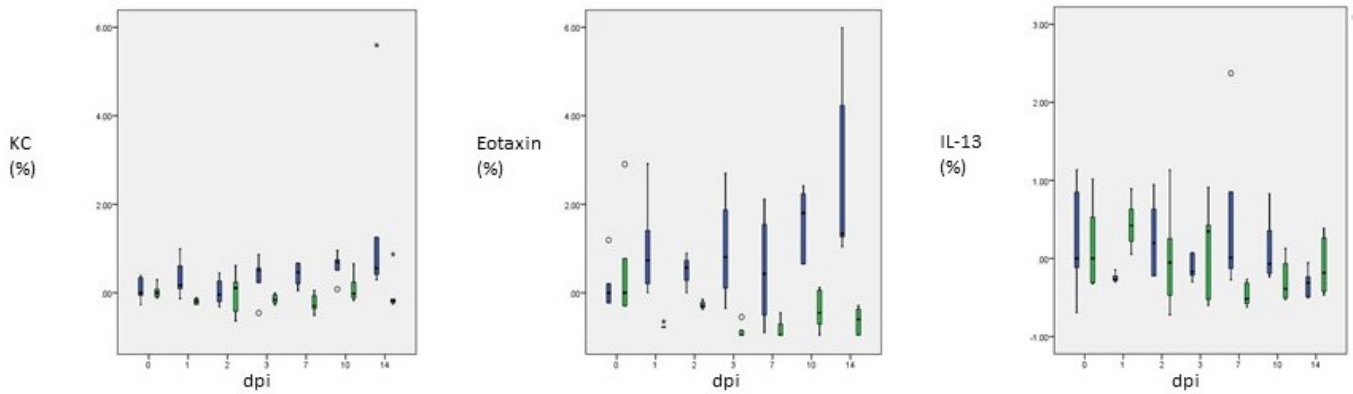

***Figure A-C5 (continued)**: Change in cytokine concentration over time for cytokines loading on principal component 5 (KC) and not loading significantly on any of the first four principal components (Eotaxin, IL-13). A baseline value was defined as the median value of group 0 for each respective cytokine. Change over time was then calculated by subtracting the baseline from individually measured cytokines and dividing them by the baseline value. Cytokine concentrations (change from baseline, 1 = +100%) are plotted on the y-axis and days post inoculation are plotted on the x-axis. Bars represent the interquartile range, medians are highlighted. Whiskers represent total variation and outliers (more than 1.5 times the interquartile range from the median) are indicated. Green: naïve group, blue: re-infected group.*

11 APPENDIX D

CULTURE WELLS DESCRIBED DURING *IN VITRO* CULTIVATION STUDIES

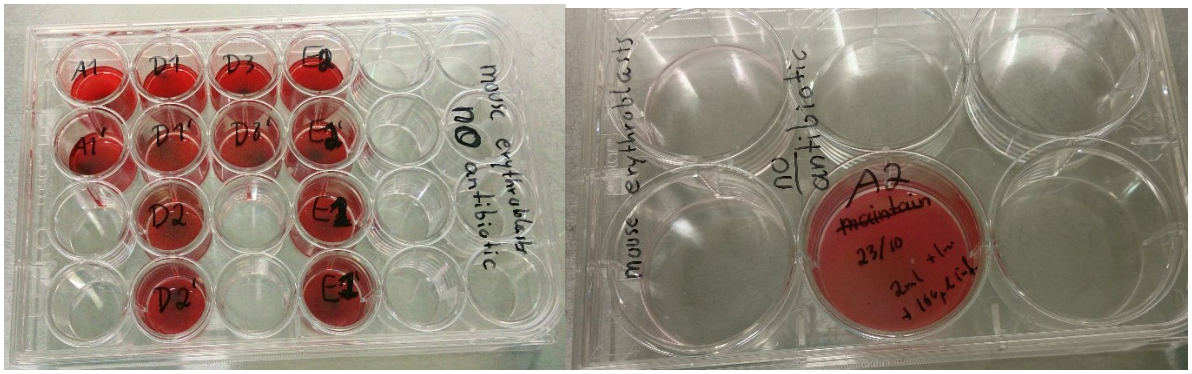

Figure A-D1 *Culture plate showing wells of the in vitro cultivation study 1, using plateau-infected blood and murine erythroblast progenitor cell lines. Duplicated wells are indicated with an apostrophe (‘). The left panel shows culture wells A1 to E2 and the right panel shows culture well A2.*

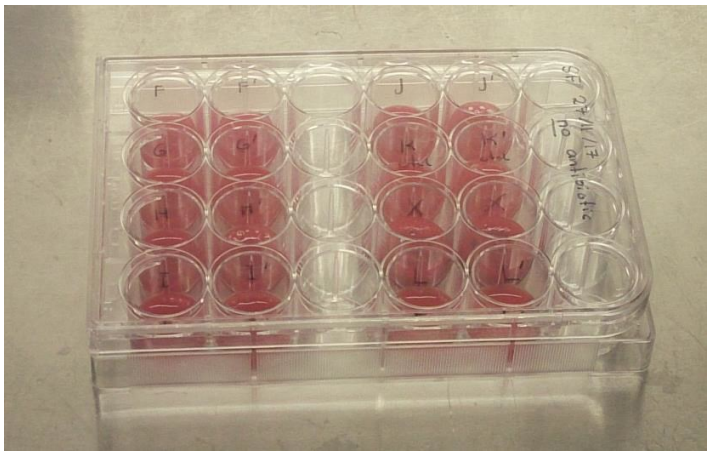

Figure A-D2 *Culture plate showing wells of the in vitro cultivation study 2, using M. haemomuris-infected blood to try to infect murine erythroblast cell lines, naïve murine blood, washed murine erythrocytes and splenocyte suspensions. Duplicated wells are indicated with an apostrophe (‘).*

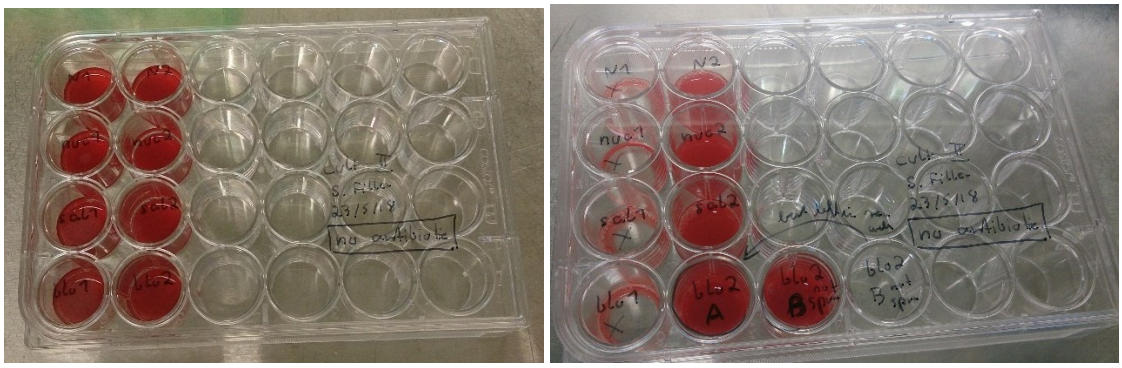

Figure A-D3 *Culture plate showing wells of the in vitro cultivation study 5 (purine/pyrimidine study). Left panel: Day of inoculation of culture wells. Right panel: Three days post inoculation of culture wells, after inoculation of half of the wells into sentinel mice and splitting well BLO2 in two identical wells supplemented with fresh mouse blood.*