University of Glasgow | Institute of Biodiversity, Animal Health & Comparative Medicine

Disease transmission and the ecological context

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This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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April 2012

Abstract

Epidemiology strongly parallels the study of ecology, primarily being concerned with the incidence, distribution, reproduction and persistence of species. The spread of disease, or its transmission, is arguably the most important incident studied in epidemiology, underpinning a pathogen's ability to reproduce and persist within a host population. However, observations of individual transmission events are often impossible to observe directly, making variation in this process difficult to study. This has resulted in a great deal of epidemiological theory being based on homogenous transmission of disease through host populations. Understanding disease transmission as a heterogeneous process requires an appreciation of the ecological dynamics determining a pathogens ability to transmit. In this thesis a cross-disciplinary approach is taken to examine the ecological dynamics that may affect disease transmission at different ecological scales.

In Chapter 2 I review empirical evidence in support of density dependent transmission. Transmission rates of density dependent transmitted diseases are often assumed to scale linearly with host population density. This assumption is pertinent to the calculation of the basic reproductive number R_0. As R_0 is important in determining optimal vaccination strategies, population thresholds and epidemic sizes, incorrect assumptions used in its calculation have the potential to misinform disease control strategies. Alarmingly, there is very little evidence to suggest that the prior assumption of a linear relationship between disease transmission rates and host population density exists. Where evidence of density dependent transmission has been found this has been best explained by non-linear relationships. Furthermore, density may have much stronger effects on disease transmission at small, local, scales (for example within one social grouping of hosts). Disease transmission between groups of hosts, at global scales, is more likely to follow frequency dependent dynamics. Disease transmission rates should thus be thought of as variable across populations that are not homogenously distributed in space, or across social structures.

In Chapter 3 a community of pathogens infecting a population of rural red foxes, *Vulpes vulpes*, is described. Foxes cadavers were collected from a private estate

in Canterbury, Kent and a combination of direct and indirect testing for disease is used to maximise the scope of disease considered as part of this community. Specifically, I examine if any of the diseases included in this study occur together, or apart, more frequently than expected by chance alone. Within the samples collected it is found that the intracellular protozoan *Toxoplasma gondii* co-occurs with the virus canine adenovirus type-I (CAV-I) more frequently than expected by chance. Foxes concomitantly infected with these pathogens have lower condition scores than foxes who were not positive for both pathogens. From the data collected it is not clear whether hosts of lower condition are more susceptible to co-infection or if the co-infection is more harmful to hosts than being singly infected. *T. gondii* is not transmitted by foxes, but if infection with this parasite increases susceptibility to CAV-I then this virus may benefit from the presence of *T. gondii* within its host population. If it is the case that foxes of lower condition are simply more prone to co-infection then it should be expected that individual differences between hosts would cause heterogeneity in disease transmission. The need for cross-disciplinary approaches when studying pathogen communities is well demonstrated by this study, as is the need for more consideration to be paid to the community ecology of pathogens in epidemiological studies.

In Chapter 4 a model is formulated to explore the effects of an interaction between a micro and a macro parasite. This is performed in the context of the increased prevalence and geographical range of the highly zoonotic small fox tapeworm *Echinococcus multilocularis* following successful rabies elimination in Western Europe. I explore the hypothesis that foxes with extremely high burdens may be at a higher risk of contracting rabies than foxes with low worm burdens, and thus rabies may have a regulatory effect on *E. multilocularis* populations by preferentially removing “super spreading” hosts. It is demonstrated that rabies limits *E. multilocularis* populations by limiting the density of available hosts. An interaction between rabies transmission rate and worm burden only caused a weak additional suppression on *E. multilocularis* populations, regardless of whether this relationship was linear or exponential. The elimination of rabies across Western Europe is certainly to be applauded. However, it should be noted from this work that surveillance of pathogen communities following successful eradication of one pathogen is of the upmost importance.

Finally, in Chapter 5 I examine how parasites adapt their investment in transmission in response to environmental changes experienced within a host. This is done by fitting models to data collected from mice infected with the malaria parasite *Plasmodium chabaudi* during the acute stage of inaction. Parasites are predicted to alter their behaviour in response to host stress, immunity and the availability of resources. However, theoretical and experimental studies reach conflicting conclusions regarding the "optimal response" to degradation of their habitat. Models were fitted to time series data from infection with one of six distinct genotypes. It is found that proportional allocation of resources into transmission, rather than replication, is highly sensitive to red blood cell (RBC) densities, with investment in transmission increasing as RBC resources become scarce. Investment in transmission also increases, albeit more weakly, in response to low parasite densities. These analyses highlight the fact that the complexity of interactions between parasites and their host hinder the identification of causal relationships, but supports recent work that questions the role of terminal investment in transmission in response to changes in the within-host environment.

The broad scope of work presented here investigates a wide range of ecological factors (including community dynamics, habitat variability and reproductive success) at different ecological scales, responsible for heterogeneity in disease transmission. Transmission is a dynamic, and heterogeneous process. To better understand the ecology of disease it is logical to investigate the mechanisms behind this variation.

Table of Contents

Abstract 1

List of Tables 7

List of Figures 8

Acknowledgements 11

Author's Declaration 14

1 General Introduction 15

1.1 The importance of understanding the ecological drivers behind variable disease transmission 16

1.2 The ecology affecting transmission acts at different scales 18

1.3 Studying disease transmission demands a cross-disciplinary approach 22

1.4 Thesis structure 23

2 A review of empirical data on density dependent transmission and its implications for the basic reproductive number 26

2.1 Introduction 26

2.2 The basic reproductive number 27

2.3 Density vs. frequency dependent transmission 28

2.4 The relationship between density and contact rate 30

2.5 The effect of experimental manipulations of host density on disease transmission 33

2.6 Evidence of density dependent transmission from naturally occurring epidemics 36

2.7 Insights from transmission networks 41

2.8 Conclusions 44

3 Exploring a disease community in a British population of rural red foxes 46

3.1 Introduction 46

3.1.1 The concept of pathogen community 46

3.1.2 The red fox as a host population 47

3.1.3 Pathogens of interest 48

3.2 Methods 50

3.2.1 Sourcing of fox cadavers 50

3.2.2 Sample collection and analysis 50

3.2.3 Data analysis 52

3.3 Results 54
3.3.1 Disease Prevalence 54
3.3.2 Co-infections 56
3.3.3 The relationship between disease and host condition 59
3.4 Discussion 60

4 A model for micro-macro parasite interactions: Could rabies regulate *Echinococcus multilocularis* populations in red foxes? 67
4.1 Introduction 67
4.2 Methods 71
4.3 Results 76
4.3.1 Does rabies virus regulate *E. multilocularis* populations through limiting host availability? 76
4.3.2 Does a linear interaction between worm burden and rabies susceptibility further limit *E. multilocularis* populations? And could it effect the over-dispersion of worms amongst hosts? 76
4.3.3 Do different forms of interaction between worm burden and rabies susceptibility differently affect *E. multilocularis* population size and over-dispersion amongst hosts? 81
4.3.4 Relaxing the assumption of a constant R_0 85
4.4 Discussion 87

5 Plasticity in transmission strategies of the malaria parasite, Plasmodium chabaudi: environmental and genetic effects 93
5.1 Introduction 93
5.2 Methods 95
5.2.1 Experimental design 97
5.2.2 Models and analysis 99
5.3 Results 102
5.3.1 Proportional investment in gametocytes in influenced by both parasite and red blood cell density 102
5.3.2 Relative investment in male gametocytes over females is influenced weakly but positively by resource availability near the peak of acute infection 106
5.4 Discussion 109
5.4.1 Mechanisms of sensing the environment 109
5.4.2 Interpretation of parasite strategies 110
5.4.3 Correlates of virulence 111
5.4.4 Top down vs. bottom up control 111
5.4.5 The timing of developmental cues 112

5.4.6 Altering sex allocation in response to RBC availability 113

6 General discussion 115

7 Appendices 121

7.1 R code for Chapter 4 121

7.2 R code for Chapter 5 127

List of References 130

List of Tables

Table 3.1 Criteria used to determine the condition score of fox cadavers. 51

Table 3.2 Summary of the collection and usage of samples collected during post-mortem examination of foxes. 52

Table 3.3 Disease prevalence in host population. 56

Table 4.1 Definitions of parameters and assigned values. RF refers to 'rabies free' state. 75

Table 4.2 Equilibrium populations of foxes and *E. Multilocularis* under rabies free and null hypothesis conditions. 76

Table 5.1 Summary of models of gametocytogenesis. The best-fitting models for all strains were of the form of equation 5.2, where *X* was total RBC or reticulocytes..... 102

Table 5.2 Modelling sex allocation across the entire infection. For some genotyopes, multiple models lay within range of 2 units of BIC and so were considered to have equivalent support. 108

List of Figures

Figure 2.1 Schematic of the assumptions behind formalising the basic reproductive number based on density dependent transmission..... 29

Figure 2.2 Schematic of the effects of heterogeneous population structure on the basic reproductive number. 36

Figure 2.3 Schematic of the effects of heterogeneous host density and allowing contacts between groups on the basic reproductive number..... 38

Figure 3.1 Detection probabilities of disease across a range of prevalences. 55

Figure 3.2 Coinfection matrix. Ticks indicate a disease pair that occurs together more frequently than expected by chance and crosses indicate those that did not..... 57

Figure 3.3 Coinfection matrix of disease parings occurring together less frequently than expected by chance. 58

Figure 3.4 Comparing condition scores of foxes that tested positive and negative for each disease..... 59

Figure 3.5 Comparing weights of foxes that tested positive and negative for each disease. 60

Figure 4.1 Variation of the rabies transmission co-efficeint β as (a) linear and (b) exponential functions of worm burden..... 73

Figure 4.2 Equilibrium population densities at varying interaction strengths when β_j is modelled as a linear function of worm burden under density dependent transmission. 77

Figure 4.3 Equilibrium population densities at varying interaction strengths when β_j is modelled as a linear function of worm burden under frequency dependent transmission. 78

Figure 4.4 Equilibrium aggregation of worms at different interaction strengths when β_j is modelled as a linear function of worm burden under density dependent rabies transmission. 79

Figure 4.5 Equilibrium aggregation of worms at different interaction strengths when beta is modelled as a linear function of worm burden with frequency dependent rabies transmission. 80

Figure 4.6 Equilibrium population densities at varying interaction strengths when β_j is modelled as an exponential function of worm burden. 81

Figure 4.7 Equilibrium population densities at varying interaction strengths when β_j is modelled as an exponential function of worm burden with frequency dependent rabies transmission. 82

Figure 4.8 Equilibrium aggregation of worms at different interaction strengths when β_j is modelled as an exponential function of worm burden with denisty dependent rabies transmission. 83

Figure 4.9 Equilibrium aggregation of worms at different interaction strengths when β_j is modelled as an exponential function of worm burden with frequency dependent rabies transmission. 83

Figure 4.11 Contour plots showing the sensitivity of fox density and *E. Multilocularis* biomass to changes in interaction strength and rabies R_0 when rabies is modelled with frequency dependent transmission. 85

Figure 4.12 Contour plots showing the sensitivity of fox density and *E. Multilocularis* biomass to changes in interaction strength and rabies R_0 when rabies is modelled with frequency dependent transmission. 86

Figure 5.1 Schematic of asexual replication and gametocytogenesis in *P. Chabaudi*. (provided courtesy of Sarah Reece) 96

Figure 5.2 Time courses of RBC and parasite densities. Data from day 5-16 PI with six genotypes of *P. Chabaudi* (AJ, AS, CR, CW, DK, ER). Longitudinal data, five mice per genotype (see methods). 98

Figure 5.3 Best fits to gametocyte density for each mouse, by strain. Y axes show the logarithm to base 10 of gametocyte densities per ml of blood..... 103

Figure 5.4 Best fits to male proportion for each mouse, by strain. 107

Acknowledgements

PhDs rarely seem to go according to plan, and mine has certainly been no exception. I've been lucky enough to have people (probably too many to mention) involved in my life over the past four years who've helped me to cope with the trials and tribulations thrown at me. I would like to thank all of the staff and students who have been part of the Graham Kerr Building over the past years for providing such a great working environment, and a supply of field assistants for my unsuccessful field work! In particular, I would like to thank those mentioned below.

My PhD was funded by the BBSRC "Organismal biology" scholarship, and I would like to thank the BBSRC for providing this opportunity. The work in Chapter 5 was carried out in New York, NY and would not have been possible without funding from the Jim Gatheral Scholarship.

I am very much indebted my two supervisors: Professor Dan Haydon and Doctor Louise Matthews. Both of whom have encouraged me to follow my scientific interests (no matter how off topic they seem to take me!) and their faith in me has certainly helped to develop me into someone who can, hopefully, be considered a well-rounded, multi-disciplined scientist. I am especially grateful to Dan who has been acting as my research supervisor ever since my undergraduate honours project back in 2006, and was instrumental in finding me funding opportunities for this PhD. Dan has always managed to maintain a positive attitude to my work, even through the start of this research project when my desire to play with the local wildlife led me on what would prove an almost two year long, completely fruitless, hunt for foxes! I couldn't have made it through the last four years without either of my supervisors, so thank you both. Doctor Roman Biek was also of great help at the start of my PhD, providing useful advice on fieldwork and allowing me to register for a personal Home Office licence under his project licence. Professor Sarah Cleaveland has also been of great help during this project, often acting as a friendly and enthusiastic sounding board for my ideas, as well as providing inspiration for some of the directions I have found my work progressing in.

I must also thank Professor Andrew Yates at the Einstein Medical Institute, New York, NY. I am very much indebted to him not only for blindly taking me on for three months as a research student, but also for giving a place to stay on arrival in New York and help getting settled into life in the Big Apple. This opportunity certainly proved to be one of the highlights during my time carrying out this PhD.

Upon starting my PhD, I carried out a lab rotation under the care of Doctor Barbara Mable with her (then) PhD student Clare Marsden. Having come from doing a Zoology degree I can honestly say I had no idea what to expect from a placement in a molecular lab, but Barbara and Clare had me carrying out my own PCRs and other "genetics stuff" (term coined by Dr. Le Vin) in no time! I am grateful for the patience awarded me in learning these techniques, which I am sure will be use to me furthering my career. I must also thank Aileen Adams for her role in helping to teach me, and providing coffee and chocolaty goodies.

Thanks to Jonathan Swire and Nigel Cox for providing (and storing) the fox carcasses used in Chapter 3. And thanks to Cathy Lamm and Caroline Millins for help with the post-mortems.

I have had the pleasure of sharing my office with a great series of people over the years: (in no particular order) Andrew Higginson, Hawthorne Beyer, Anaid Diaz, Sunny Townsend, Douglas Kerlin, Elizabeth Masden, Meggan Craft, Grant Hopcroft, Rob Critchlow, Victoria Paterson, Dan Erben, Maganga Sambo, and finally my partner in crime, Flavie Vial (Couldn't have finished this without you, high five!). Being in an office with others willing to share and discuss ideas, and where one person's weakness has been another's strength has been tremendously rewarding, and played a huge role in making this PhD a positive and worthwhile experience. I would also like to specifically thank those who took part in our little stats club: Grant, Sunny, Meggan and Eliza. Working through our mixed effects models book turned out to be a hugely rewarding and enjoyable experience that has definitely given me a much deeper appreciation and ability in statistical analysis.

Of course there were also those who existed outside the realms of my office who have provided help and support: To my ex- flatmates Maria Rasmussen and

Kenny Armstrong, I have to say thank you for being so understanding of the odd hours I had to keep when trapping foxes. Thank you to Wesley Burnside for all the cooked meals while I was trapping foxes also! And to Cat O'Connor… Where to start?! Providing distracting emails about movies we've seen, providing a friendly ear when I've needed to vent about our mutual supervisor, being my partner in creating the (highly successful) "Infectious Matters", walking my dog when I've had to drive across the country to pick up dead foxes… you've been an absolute legend!

Special thanks must be made to the "Brain Trust": Andy Tedder, Ashley Le Vin and Flavie Vial (Team Blue!). You three have truly become some of my closest friends. Without you I never would have experienced so many of the burgers Glasgow's West End has to offer, stalked a basketball team, solved a multitude of murders and probably wouldn't cook such amazing spag bol. I am eternally grateful to all three of you for making the last four years "dead good"!

I thank my long-suffering parents (especially you Mum) for putting up with my stress-induced mood swings and long rants on the phone about how "life isn't fair" and "I'm never going to finish my PhD". Thank you for believing in me.

And finally to Wes, the most important person in my life. Your love and support have kept me going and allowed me to finish this thesis with (at least a little of) my sanity intact.

I dedicate this thesis to my four amazing grandparents: Bill and Margaret Cameron; and Ken and Pauline Holmes.

Author's Declaration

I declare the work recorded in this thesis is entirely my own, except where otherwise stated, and that it is also of my own composition. Much of the material included in this thesis has been produced in co-authorship with others, and my personal contribution to each is as follows:

Chapter 2. Cameron, A., Matthews, L., and Haydon, D.T. A review on the empirical data on density dependent transmission and its implications for the basic reproductive number. Initial concept developed by DTH. Literature search and chapter drafted by AC. Final draft enhanced by DTH and LM.

Chapter 3. Cameron, A., Lamb, C., Matthews, L., and Haydon, D.T. Exploring a disease community in a British population of rural red foxes. Initial concept developed by AC. Sample collection from animals post-mortem by AC and CL. Histo-pathological analysis of tissue samples by CL. Data analysis conducted and chapter draft by AC. Final draft enhanced by DTH, CL and LM.

Chapter 4. *In preparation for submission as*: Cameron, A., Matthews, L., and Haydon, D.T. A model for micro-macro parasite interactions: Could rabies regulate *Echinococcus multilocularis* populations in red foxes? Initial concept developed by AC. Model formulation by AC, LM and DTH. Model simulation and chapter drafted by AC. Final draft enhanced by LM and DTH.

Chapter 5. *Submitted as*: Cameron, A., Reece, S., Drew, D., Haydon, D.T., and Yates, A.J. Plasticity in transmission of the malaria parasite, *Plasmodium chabaudi*: environmental and genetic effects (submitted to *Evolutionary applications*). Data collected and provided by SR and DD. Initial concept developed by AJY, SR and AC. Model formulation by AC and AJY. Data analysis conducted and manuscript drafted by AC and AJY. Final draft enhanced by SR and DTH.

I further declare that no part of this work has been submitted as part of any other degree.

Angus Cameron April 2012

1 General Introduction

Epidemiology has, at its heart, much of the same underlying philosophy as ecology. The parallels between the two fields are clear, with both being concerned with the incidence, distribution, spread and persistence of species. The spread of a disease, or its transmission, is arguably the most important process studied in epidemiology, underpinning the ability of a pathogen to persist in a host population and determining the dynamics of epidemics. However, the transmission between individual hosts often cannot be observed directly, making variation in this process difficult to study. This has resulted in traditional mathematical models treating transmission as a homogeneous phenomenon, which a great deal of modern epidemiological theory has been based on. To understand transmission as a heterogeneous phenomenon requires consideration of the habitats pathogens invade, their hosts, and the interactions that both host and pathogen are involved with in their environment.

Heterogeneous disease transmission was recognised as early as 1907 when an Irish cook, Mary Mallon, was implicated as being responsible for 51 cases of typhoid in a seven year period in New York – despite never showing symptoms of disease herself (Stein 2011). The infamously coined "*Typhoid Mary*" was recognised as an asymptomatic carrier of typhoid and confined by New York health officials between 1907 and 1910. Upon release however she went on to become a cook at two hospitals, where a total of more than 200 patients were affected before she was again discovered, and isolated for the remainder of her life (Nester et al. 1973).

Despite such an infamous case of variable transmission in history, modern epidemiological thinking has been driven to a large extent by the seminal works of Anderson and May throughout the 1970s and 80s (Anderson & May 1979; Anderson & May 1982; Anderson & May 1985; May & Anderson 1979). The epidemic models proposed by Anderson and May have undeniably proven invaluable in predicting and curtailing epidemics; explaining why some diseases are endemic or cyclic; and furthering our understanding of host population factors that may facilitate or hinder transmission (Anderson & May 1991b).

However, in their simplest and most widely read and understood form these traditional epidemiological models assume homogenously distributed, homogenously infectious and susceptible individuals with overall transmission dynamics governed by a single parameter, β. It seems unlikely this was Anderson and May's intention, as they have formulated several models capable of exploring heterogeneity in transmission between individuals (Agur et al. 1993; Anderson & May 1988; Anderson & May 1991b; May & Anderson 1984). However, the appeal of their simple transmission models has been great and lead to them being used, largely, without question. Rather than being fixed, transmission should be considered as a dynamic trait of pathogens; a trait that will be dependent on pathogen fitness, genetic variability, and forces acting both within and between hosts (Antolin 2008; Real & Biek 2007).

1.1 The importance of understanding the ecological drivers behind variable disease transmission

Human populations currently face an unprecedented rate of emerging and re-emerging disease (Cleaveland et al. 2007; Woolhouse 2002). Anthropogenic induced changes in natural environments and climate change have been proposed as underlying causes of this phenomenon. Resulting in increased disease incidence, changes in geographic range of vectors and invasion of new host species (Schrag & Wiener 1995; Woolhouse 2011; Yacoub et al. 2011). Historic disease emergence events have also been linked to anthropogenic processes, primarily the domestication of wild plants and animals (Diamond 2002; Pearce-Duvet 2006). Zoonoses account for 60% of human infectious disease burden, and about 70% of emerging disease pathogen "species" (Taylor et al. 2001), implicating transmission between humans, wildlife and livestock as an important component in disease emergence. To better understand the factors responsible for disease emergence and pathogen transmission across species barriers requires an understanding of how pathogen transmission is mediated by changes in the environment that pathogens inhabit. This will be essential in predicting the impact of future environmental change and habitat degradation on threats posed by disease.

Emerging diseases are not exclusively of human concern. We are currently in the midst of a mass extinction event (Dunn et al. 2009; Wake & Vredenburg 2008),

with many wildlife populations only persisting in small, fragmented populations. Historically, disease has been an uncommon cause of extinction, with habitat loss, climate change, over-exploitation and the introduction of alien species having a more profound impact on the long-term viability of endangered populations (Pimm et al. 1995; Purvis et al. 2000; Wilcove et al. 1998). However, the short-term persistence of some endangered populations is at immediate risk in the face of emerging diseases (Haydon et al. 2006; Pedersen et al. 2007; Prager et al. 2011; Smith et al. 2006; Vial et al. 2006; Wake & Vredenburg 2008). It should be noted that mass extinction itself has been proposed as a driver of disease emergence through loss of biodiversity reducing the "dilution effect" where the presence of incompetent hosts can help reduce transmission opportunities to competent hosts (Schmidt & Ostfeld 2001) or by removing genetic variation present in host populations leading to less variation in host immune systems (Maillard & Gonzalez 2006).

Small populations do not generally act as reservoirs for disease, and pathogens may fail to successfully invade host populations when they are smaller than the critical community size (CCS) (Lloyd-Smith et al. 2005). This is predicted to make small populations robust to limited disease introductions, as epidemics must "burnout" when populations become too small (or too sparse) to maintain an epidemic (Lloyd-Smith et al. 2005; McCallum et al. 2001; Smith et al. 2006). However, as humans encroach further upon wildlife habitats there is increased potential for multiple disease introduction events into endangered populations from domestic animals (Cleaveland et al. 2001), and forcing wild populations into closer contact may allow multiple smaller populations to act as a single multi-species reservoir (Haydon et al. 2002). Where a population is too small to propagate an epidemic, it is the frequency of transmissions into this population that determines the severity of disease induced population declines (de Castro & Bolker 2005). The synchronicity of these introductions may also affect the impact a disease has on an endangered population; it may be easier for a small population to survive against a low, but constant rate of disease introduction rather than a high frequency of introductions across shorter time scales; unless disease is directly responsible for constraining the population to below CCS, in which case breaks in disease introduction would allow the host population size to increase leading to bigger epidemics. The ability of a pathogen to transmit

between host species and in small or sparse populations is fundamental to the conservation threat a disease poses. Understanding the ecology that hinders or facilitates these processes is necessary for the design of effective disease control strategies, and monitoring their success.

Heterogeneity in disease transmission can have profound effects on the epidemiology of a disease and the effectiveness of control or eradication efforts. Many disease systems would appear to conform to the 20/80 rule: whereby 80% of the transmission potential of disease is held by only 20% of infected hosts (Woolhouse et al. 1997). This transmission heterogeneity is often caused by aggregation of pathogens, where the majority of infected hosts are only lightly infected, but a minority of hosts carries very heavy burdens of infectious material (Guislain et al. 2008; Woolhouse et al. 1997). However the underlying cause of this phenomenon is unclear, and in reality is likely to differ dependent on the host pathogen system in question. Proposed reasons include increased strain virulence (Matthews et al. 2009), a higher propensity of the host to shed (Stein 2011), and co-infection with other pathogens (Bassetti et al. 2005a; Bassetti et al. 2005b). These proposals can all be considered ecological in nature, whether due to different genotypes' abilities to exploit their environment, or through community interactions with other species. The presence of super-spreaders has obvious implications for disease control strategies: strategies that can target this core 20% of super-spreading hosts will be highly efficient, and effective and conversely those that fail to treat this 20% will be costly and ineffective (Matthews et al. 2006; Woolhouse et al. 1997). Heterogeneity in transmission also increases the basic reproductive number, R_0, of a pathogen (Woolhouse et al. 1997). This would be predicted to lead to faster disease spread through a host population and larger epidemic sizes than predicted (Tildesley & Keeling 2009).Treating transmission as homogenous could thus lead to under-estimating the threat posed by a novel pathogen introduced to a susceptible population.

1.2 The ecology affecting transmission acts at different scales

Pathogens co-exist in complex and dynamic environments, where their survival and investment in transmission is affected by changes in their immediate

habitat, within the host. Future transmission and persistence are affected by changes in the ecology and population structure of their hosts (Anderson & May 1991b; Bull 1994; McCallum et al. 2001; Pedersen & Fenton 2007). Clearly, the ecology affecting disease transmission is dependent on the scale at which it is considered. Bush and Holmes (1986) define three scales at which pathogen communities may be examined: the *infra community* consists of the pathogens infecting a single host simultaneously; the *component community* is made up of the pathogens infecting a population of hosts at any time; finally *compound communities* consist of the pathogens present in a community of hosts. These scales of community are also useful when considering other aspects of disease ecology. At each of these three scales the ecological pressures faced by pathogens have the potential to affect transmission dynamics.

At the scale of the infra community, changes in a pathogen's immediate habitat, within the host, have an effect on transmission from one host to the next. This can be with respect to the host's immune system (Graham 2002; Graham et al. 2007), the community with which it shares it's host (Cox 2001; Graham 2008; Hawley & Altizer 2011; Jolles et al. 2008), and changes in availability of resources (Pedersen & Fenton 2007). These within-host factors are not mutually exclusive, and interplays between them may prove important in determining a pathogens ability, and disposition to invest in transmission (Haydon et al. 2003). For example the immune system may play an important role in the competitive dynamics between pathogens within a host (Cox 2001; Graham 2002). T-cell polarisation is a process of the adaptive immune response of mammals with two mutually exclusive outcomes; either a Th1 or Th2 response is elicited based on the cytokine profile stimulated by infection. Generally the Th1 polarisation occurs in the presence of micro-parasites and Th2 polarisation in response to macro-parasites. T-cells are a limited resource, and when one "arm" of this response is up-regulated, the other must be down-regulated (Cox 2001). This dynamic trade-off has been demonstrated to influence the competitive, or synergistic response and pathogenesis of some parasites (Petney & Andrews 1998), and it may also be the case that differences in immune response between single and concomitant infections lead parasites to adjust their investment into transmission.

Within an infra community it may also be expected that the influence of resource utilisation on virulence would affect the transmission strategy employed by pathogens (Bull 1994). Pathogens that rapidly consume resources do so at the cost of causing virulence to the host. In extreme cases this ends the infectious period by killing the host. Pathogens exhibiting this extreme strategy must have very efficient transmission to maximise their probability of successful transmission over a limited period of time. Pathogens may also utilise their host more sustainably, causing minimal virulence over much longer periods of time. This strategy usually results in a lower probability of transmission over longer periods of time (Alizon & van Baalen 2008). These strategies should be considered as opposite ends of a continuum, both of which aim to maximise R_0. The position along which a pathogen lies will depend on host immunity, resource availability and interactions with other members of the infra community.

At the scale of a component community, the individual differences between pathogen ecology at the level of infra communities manifest as heterogeneity in transmission rates (Pedersen & Fenton 2007; Stein 2011; Woolhouse et al. 1997) with unpredictable outcomes: Super-spreader individuals can proliferate epidemics (Fujie & Odagaki 2007), but heterogeneity in infectiousness and susceptibility can also make the initial emergence of disease less certain (Yates et al. 2006). If disease is endemic however, targetting super-spreading individuals could make disease control more effective (Matthews et al. 2009).

Ecological factors at the component community scale will affect the transmission dynamics throughout the host population and thus the persistence of disease. The ecology of disease transmission at this scale is subject to variation caused by host behaviour, demography, seasonality, and spatial structure of populations. Pathogens may be subject to density or frequency dependent transmission, or an intermediate between these two extremes. Increases in host density could be the result of reduced top-down effects or increased bottom-up effects on the population; it may also be sensitive to anthropogenic interference of host habitat. The behaviour of hosts can lead to more frequent contacts between individuals, leading to an increased potential for disease transmission; for example through a high number of sexual contacts (Ji et al. 2005) or fighting for a position in a social hierarchy (Beisner & Isbell 2011). Seasonal forces can also strongly affect the transmission dynamics of

disease. A host's behaviour in response to seasonal changes may affect disease dynamics, for example if contact rates are higher during breeding seasons (Tersago et al. 2011) or seasonal migrations may provide opportunities for disease introductions to naïve populations (Lawson et al. 2012; Morgan et al. 2006).

The ecological factors affecting pathogens in a component community also affect those at the compound community level. However, there are additional ecological factors to consider at this level, especially for diseases that are not directly transmitted. For instance, predator-prey relationships may influence the transmission dynamics of trophically transmitted pathogens: parasites that utilise the predator-prey relationship between definitive and intermediate hosts for transmission. Parasites themselves may also have their transmission potential lowered by predation if ingested by a non-competent predator, halting the spread of infection (Johnson et al. 2010). Transmission dynamics of trophically transmitted pathogens may be further affected by changes in the behaviour of intermediate hosts (Parker et al. 2009). For example *Toxoplasma gondii* causes reduced neophobia (Webster et al. 1994) and sexual arousal by cat urine odours (House et al. 2011) in infected rodents. In this example a population of mice with heavy *T. gondii* infection may suffer a greater predation from cats, but this would also have a direct effect on other pathogens in the mouse population whose hosts may now suffer increased predation by a potentially non-competent host. The increased infectious load of *T. gondii* may affect pathogens in the cat population too if it interacts with concomitant pathogens at the infra community level. Predator prey relationships may also affect pathogen communities when predators show preference for the sickest, or healthiest prey (Hall et al. 2005). Predator prey dynamics can be complex and are important in the maintenance of "healthy herds" (Packer et al. 2003), whereby predation may reduce disease prevalence in their prey and also facilitate prey population growth. At this scale disease may also facilitate the invasion by alien species, even if they are poorer competitors than native species, so long as they are less negatively affected by native pathogens (Bell et al. 2009). If the alien species manages to out-compete the native host population this may also have knock-on effects for the native pathogens that facilitated the invasion.

The ecological dynamics acting at each of these scales have the potential to be extremely complex, and should not be expected to act independently from one another either within or across the scales outlined here. However, classifying disease ecology by the scale at which pathogens interact with the environment provides a natural and intuitive way to divide the "big picture" into manageable pieces.

1.3 Studying disease transmission demands a cross-disciplinary approach

There are clearly several approaches and disciplines required to consider the ecological forces experienced by pathogens both within and between the scales described above. To effectively study the interactions between pathogen, host and environment disease ecologists must integrate ecology across a variety of fields including: immunology, microbiology, parasitology, physiology, population biology, genetics and community ecology all within an epidemiological framework (Archie et al. 2009; Hawley & Altizer 2011; Pedersen & Fenton 2007; Petney & Andrews 1998).

Realistically, an entirely holistic approach encompassing aspects from such a broad spectrum of disciplines is likely beyond the abilities of any single ecologist or epidemiologist. Again, by breaking up the ecological drivers affecting disease transmission by the scale on which they act provides an intuitive and manageable way to class research questions by. The necessary tools for disease ecologists working at different scales shall also come from different disciplines.

Cross-disciplinary studies into disease ecology have already proven important for disentangling complex disease dynamics that traditional epidemiological processes have struggled to explain, for example: By combining molecular, behavioural and epidemiological methods the reservoir dynamics, persistence and importance of inter-species transmission of multi-host pathogens have been explored in the Serengeti eco-system (Craft et al. 2008; Craft et al. 2009; Lembo et al. 2008); By considering the host immune system as a "top-down pressure" and the variation and limitations of the immune responses to different, and multiple pathogens a better understanding of the facultative and competitive dynamics of pathogens in co-infection has been achieved (Cox 2001; Graham

2002; Graham 2008; Hawley & Altizer 2011; Jolles et al. 2008); The application of evolutionary and life history trade-off theories examining parasite investment in growth and reproduction is beginning to shed light on the ability of parasites to adaptively alter their infection dynamics in response to competition, resource availability, and inbreeding avoidance (Mideo & Day 2008; Mideo & Reece 2012; Pollitt et al. 2011a; Pollitt et al. 2011b; Reece et al. 2010; Reece et al. 2008; Reece et al. 2003; Reece et al. 2009). These examples demonstrate the value in cross-disciplinary approaches to studying disease ecology. To understand the ecological dynamics affecting disease transmission at different scales such cross-disciplinary techniques will be vital.

1.4 Thesis structure

The recognition that disease transmission is not a homogenous process lies at the heart of this thesis. A broad spectrum of topics is covered bearing this in mind. The ultimate goal of this body of work is to gain a better understanding of how host and pathogen ecology at different scales relates to heterogeneous disease transmission and their epidemiological consequences. My research strategy has included:

1. Demonstrating the importance of analytical approaches integrating both empirical and theoretical methodologies.

2. Evaluating the relationship between host density and disease transmission.

3. Examining the composition of component communities, the frequency of co-infection and its relation to host health.

4. Theoretically exploring how pathogens with differing transmission strategies may compete for hosts and the epidemiological consequences arising from this.

5. Understanding how the transmission strategy employed by a parasite may vary with respect to changes occurring in the environment experienced within a host.

Traditional epidemiological research has focused on single host, single pathogen systems with homogenous disease transmission assumed. This research demonstrates the importance of treating transmission as a dynamic and heterogeneous process as well as demonstrating the need to consider the potential interactions between pathogens in a community when studying their epidemiology.

In **Chapter 2**, a review of literature that provides empirical evidence for density dependent transmission is made. Specifically, I look for evidence of pathogens whose basic reproductive number, R_0, increases at higher host densities. The review then goes on to discuss the relevance of global host density to R_0, or if local density should be expected to have a bigger impact.

The composition of a component community of pathogens infecting a rural red fox population is examined in **Chapter 3**. By making use of direct and indirect testing it was possible to consider a wide range of pathogens (including protozoa, viruses and bacteria) that may be present in this host population. I examine combinations of disease occurrence in this population and look for disease pairings that occur together or apart more than expected by chance alone, as this could be indicative of facilitative co-infection, or competitive exclusion relationships.

A theoretical exploration of a competitive interaction between a micro and a macro-parasite is made in **Chapter 4**. Specifically, I investigate the potential for rabies virus to regulate the cestode *Echinococcus multilocularis* in red fox populations. I consider the effect of an interaction where foxes "super-infected" with *E. multilocularis* are more likely to contract rabies, and the implications this would have for total *E. multilocularis* burdens following large-scale rabies eradication programmes in Western Europe.

In **Chapter 5** models are developed to describe how parasites alter their investment in transmission as a function of changes experienced in the within-host environment. Time-series data collected from mice during the acute phase of infection with the malaria parasite, *Plasmodium chabaudi*, are analysed to determine if six genetically distinct clones alter their investment in producing gametocytes necessary for transmission. The models used quantify the

importance of resource availability and density dependence to the trade-off experienced between asexual growth (replication) and sexual reproduction (transmission).

The importance of studying disease transmission in an ecological context, as a dynamic and variable process, is discussed in **Chapter** 6. Understanding the mechanisms responsible for heterogeneity in disease transmission is vital given the severity of threats posed by emerging disease to humans, their livestock and endangered wildlife. Advancing knowledge of these mechanisms will be important for designing effective disease control strategies and also evaluating their impact on disease communities. Ultimately, understanding the ecology behind variation in transmission may also prove important in determining why, and when pathogens cross species barriers – particularly when they become zoonotic.

2 A review of empirical data on density dependent transmission and its implications for the basic reproductive number

2.1 Introduction

There has been much controversy over the past 10-20 years on "how best to model pathogen transmission" (Begon et al. 2002; De Jong et al. 1995; Lloyd-Smith et al. 2005; McCallum et al. 2001). This has largely stemmed from arguments concerning the formulation of the basic reproductive number, R_0, and how this depends on the density of available hosts. Yet the effects of population density, rather than population size, are arguably still poorly understood. Measures of population size do not encompass any measure of the spatial distribution of hosts and do not vary as a function of the area a population inhabits. Measures of population density are defined as the number of individuals per unit of space, and are thus concerned with the spatial organisation of a population. This will vary as a function of the area a population occupies. Measures of density will also differ dependent on the scale at which it is considered: The density of individuals at a local scale, for example within a single social unit (a family, or a pack) may be much higher than the global population density as individuals within this unit spend large proportions of their time in close contact with one another. This may be thought of as heterogeneity in density throughout the area a population occupies. Traditionally, host density is considered homogenous in epidemiological models (Anderson & May 1982; Anderson & May 1991b; Begon et al. 2002), and the effect of heterogeneous host density on disease transmission dynamics is poorly understood.

Confusion over the separate roles of population size and density, can largely be attributed to the introduction of the term "pseudo mass action" by de Jong et al (1995), which is used to describe pathogen transmission in relation to the product of the *numbers* of susceptible and infectious hosts in a population. True mass action, in comparison, describes transmission in relation to the product of *densities* of susceptible and infectious hosts. These two modes of transmission form opposite ends of a continuum, and are better described by the terms

frequency and *density* dependent transmission. The point along this continuum at which a pathogen occurs will depend on its mode of transmission, the species that it infects and the population structure of its host.

Whilst there are substantial bodies of theory concerning the differences between frequency and density dependent transmission (Anderson & May 1982; Begon et al. 2002; De Jong et al. 1995; May & Anderson 1984; Ryder et al. 2007; Turner et al. 2003), there has been little done to confront theory concerning the effects of population density on disease transmission with empirical data.

Through a review of previous experimental and observational studies the role host density plays in influencing disease transmission is here explored. Literature searches were performed in ISI Web of Science, with combinations of the search term "density" with one or more of the following: "transmission, mass action, contact rate, R-0, R0, basic reproductive number, epidemic, network theory, contact network and transmission network". Considering papers from 1970 to present By primarily considering the empirical studies turned up by these searches we examine the effects of host density on contact rates between individuals, the influence of this on the spread of disease and the impact of this on assumptions made in calculating R_0.

2.2 The basic reproductive number

The basic reproductive number (R_0) is defined as the expected number of secondary infections occurring on average upon introduction of a single infected individual in an otherwise entirely susceptible population (Anderson & May 1991b). This concept is core to modern epidemiology, being used to predict the impact of epidemics on a host population and in designing the necessary interventions to limit them, as well as population thresholds for disease invasion and persistence (Lloyd-Smith et al. 2005; Swinton et al. 1998).

R_0 is dependent on the contact rate between hosts, the probability that a contact will be with an infected individual, and the probability that such a contact results in successful transmission (De Jong et al. 1995). Classic theoretical studies predict the contact rate between individuals to increase with population density due to random movement and homogenous mixing (McCallum

et al. 2001). These are clearly very strong assumptions, and the movements and distribution of real populations should certainly not be expected to follow them. However the question is not one of how strictly do populations follow the patterns laid out by these assumptions, but rather are these assumptions importantly wrong in describing the spread of disease.

R_0 is difficult to measure directly (but see Hampson et al. 2009), and is generally calculated mathematically by analysing epidemiological data (Breban et al. 2007; Heesterbeek 2002). When pathogens exhibit density dependent transmission dynamics this should be evident by an increased R_0 in populations with higher densities.

2.3 Density vs. frequency dependent transmission

Density dependent transmission predicts that transmission dynamics are governed by the product of the densities of susceptible and infective individuals within a well mixed, homogenously distributed population, as formalised by equation 2.1.

$$\frac{dI}{dt} = \beta SI - \gamma I \qquad \textbf{Equation 2.1}$$

Where the rate of change of infective individuals, *I*, changes linearly as a function of the transmission coefficient, β, and the densities of infective and susceptible individuals, *S*. Infected individuals recover at a rate of γ. Where disease is spread by contact events that scale positively with population density these transmission dynamics are expected.

Frequency dependent transmission predicts that transmission dynamics are independent of the densities of susceptible and infected hosts. The rate of change of infected individuals is thus governed by equation 2.2.

$$\frac{dI}{dt} = \beta \frac{SI}{N} - \gamma I \qquad \textbf{Equation 2.2}$$

The difference compared with equation 2.1 is the densities of susceptible and infected individuals are over total population density. This eliminates the effect

of density upon transmission, which now scales with the proportion of infected individuals within the population. Generally frequency dependent transmission is used to describe disease that is spread by contact events independent of density, for example sexually transmitted infections.

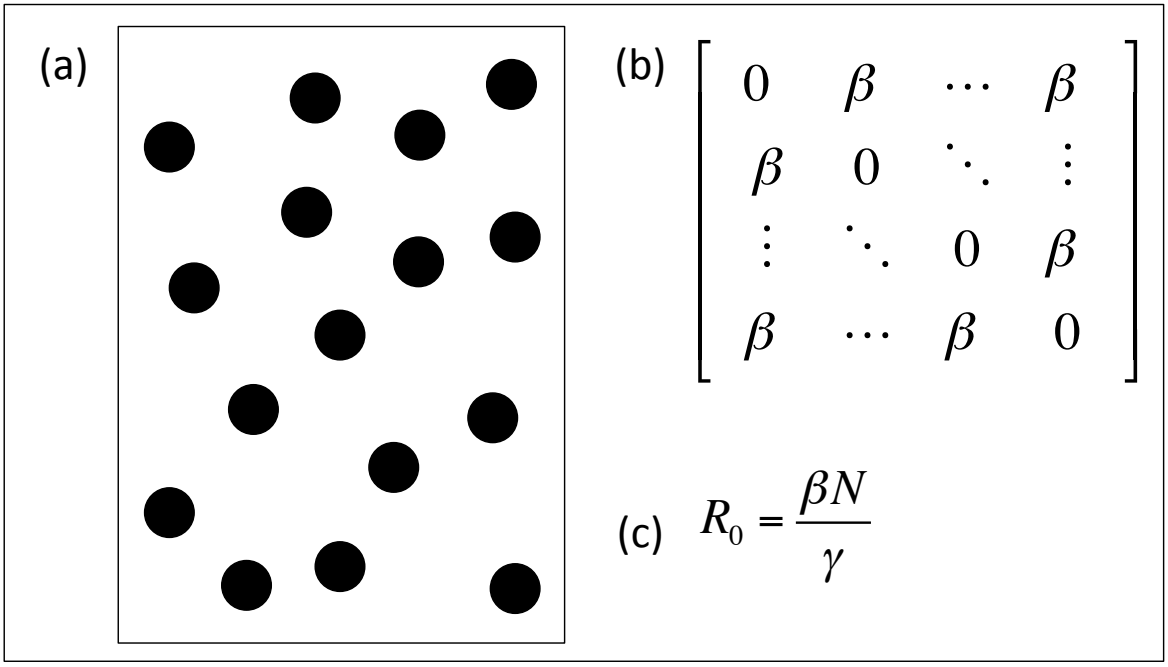

Figure 2.1 Schematic of the assumptions behind formalising the basic reproductive number based on density dependent transmission. Disease spread between individuals of a homogenously distributed, randomly moving population (a) can be described by matrix (b). Assuming transmission rates are equal between all individuals R_0 is described by equation (c).

By parameterising equations 2.1 and 2.2 with $I = 1$ and $S = N$ we can represent the scenario where a single infective individual is introduced into an entirely susceptible population. Solving for the number of secondary infections caused by the primary infected individual leads to formulations for R_0. Equations 2.3 and 2.4 are the formulae for R_0 in density dependent and frequency dependent systems respectively (assuming a closed population with no reproduction or natural mortality).

$$R_0 = \frac{\beta N}{\gamma} \qquad \textbf{Equation 2.3}$$

$$R_0 = \frac{\beta}{\gamma} \qquad \textbf{Equation 2.4}$$

From these equations it can be seen that, in theory, R_0 is predicted to scale linearly with host population density if transmission is governed by density dependent dynamics. Figure 2.1 demonstrates some important underlying assumptions of this formulation: Transmission rates between all hosts are equal; hosts are homogenously distributed and move randomly; and R_0 increases linearly with the global population density.

2.4 The relationship between density and contact rate

In its simplest terms, density is the measure of individuals per unit of area. This may be measured locally within a colony or social group, or globally over the entire range of a population. For disease transmission local densities will undoubtedly be most important, while densities measured on larger scales may have implications for disease persistence. In mathematical models of disease spread a major assumption is often that the host population is homogenously distributed in space. In practice this is seldom the case making density inherently difficult to quantify. The simplest starting point to this problem is to consider sessile organisms. Plants are hosts for which densities can be easily measured, and in the case of many agricultural species, may even conform to the assumption of being homogenously distributed in space. Burdon and Chilvers (1982) consider the measurement of crop density in their review of plant disease ecology, and discuss calculations of separation distance (a variant of local density). Assuming a homogenous distribution of hosts within a square plot leads to a separation distance given by the inverse of the square root of host density (equation 2.5).

$$L = \frac{1}{\sqrt{D}} \qquad \textbf{Equation 2.5}$$

For sessile hosts this may be considered as a “pseudo-contact rate” as density, *D*, increases the separation distance, *L*, decreases exponentially. Shorter separation distances are paralleled by the increased contact rate expected to occur in well-mixed mobile populations with increases in density. The importance of heterogeneity in separation distances is also noted when plants are “clumped”, which could be taken as analogous to heterogeneity in contact rates. Subsequent studies have demonstrated that incidence of tomato spotted

wilt virus and pea root rot disease declines exponentially as distance from infected plants increases in crop species (Coutts et al. 2004; Willocquet et al. 2007). Similar relationships have been found for plant moulds of the genus *Phytophthora* (Ristaino & Gumpertz 2000) which are transmitted by a range of mechanisms including insect vectors, soil-borne transmission and through water splashes. If disease transmission between sessile hosts in homogenously distributed populations does not change linearly with separation distance, There is no reason to expect transmission rates in mobile, heterogeneous populations to vary as a linear function of density. In fact, conflicting factors such as behaviour and sociality will likely complicate this relationship even further as contact rates between hosts will be variable. Thus the *a priori* assumption of a linear relationship between population density and R_0 is likely to prove inadequate.

Density estimates of mobile hosts vary greatly depending on the scale of which density is being measured. A single density estimate based on the entire range of a population cannot be expected to account for patches of high and low density throughout this range. For immediate disease transmission between individuals local densities should prove more important, and metapopulation dynamics of disease spread may best describe epidemics (Beyer et al. 2011). A variety of methods have been developed to calculate densities of mobile animals in their natural habitats, but most can be classed broadly as capture-recapture, distance sampling or as indirect methods (Krebs et al. 2011; Moore & Barlow 2011). Most incorporate some measure of "detection probability", which will attempt to account for individuals that were present and yet not detected. Both types of method suffer from difficulties in calculating the size of area being sampled: traps inevitably sample animals from beyond the area they physically occupy, and distance sampling methods will unlikely sample all directions evenly due to variability in landscape – so even if the area being observed was accurately measured, the area observed is near impossible to quantify.

Whilst estimating absolute densities of wild populations is undoubtedly difficult, temporal changes in densities can certainly be measured. Ji et al (2005) monitored brushtail possum, *Trichosurus vulpecula*, population densities in New Zealand from 1999 until 2001 using capture-recapture techniques. By fitting data-loggers to caught individuals contact rates between tagged individuals were

also monitored. Contact rates varied most strongly with season, being highest in the breeding season. No linear relationship between population densities and contact rates was found, and contact rates only significantly increased in response to a large influx (when population of reproductively active males doubled in 2001) of new individuals at one study site. This increase was attributed to a perturbation of the population away from its stable equilibrium size, causing changes in host contact rates through territorial and mating behaviours. In this instance, there was certainly no linear relationship between the contact rates and densities at different study sites. It may be the case that a non-linear relationship could explain the increase in contact rates in response to large perturbations in population density.

Density of mobile hosts can be controlled when kept in enclosures. Caperos et al. (2011) compared contacts of cotton-top tamarins, *Saguinus oedipus*, in large and small groups, kept in crowded and non-crowded conditions. Individuals in large groups kept in crowded conditions spent more time involved in aggressive encounters than those housed in less crowded groups. Whilst contact behaviour of the breeding pair within groups was not affected by density, the percentage of time that offspring (all other members of the groups) spent in contact with other individuals was higher when groups were kept in crowded enclosures. A study on Rhesus macaques further demonstrates the complexities of how population densities affect contact rates in primates. Beisner and Isbell (2011) modified group size in identically sized enclosures and found that displacement encounters were more frequent in higher density groups, but acts of moderate and extreme aggression were unaffected. Whilst displacement acts were the most common behaviour recorded, social complexities may result in nonlinear relationships between contact rate and density. Therefore not all types of contact between animals were equally affected by density, and not all individuals were equally affected. This demonstrates that even at local scales, density should not be assumed to linearly affect contact rates.

Despite not explicitly demonstrating a relationship between density and disease transmission the above examples highlight findings relevant to the relationship between density and contact rates of mobile hosts: While higher density populations may exhibit higher contact rates, this increase is not merely attributed to random mixing of populations. It will be dependent on the

behaviour of individuals and the social groupings and hierarchies present in a population. Consequently, contact rates (and therefore R_0) should not necessarily be expected to vary as a simple linear function of host population density.

2.5 The effect of experimental manipulations of host density on disease transmission

Contact rate forms only one component of the force of infection. Demonstrating true density dependent transmission requires establishing that the increases in contact rate actually result in more infections following introduction of an infected individual into a population. Experimental manipulation of host density should allow the impact on disease transmission to be quantified.

Livestock, in theory, provide an excellent experimental model: they can be confined in pens where the area can be easily measured, and the number of animals within a pen (and thus density of animals) can easily be controlled. A study examining pseudorabies virus (PRV) in pigs failed to show that transmission varies in populations of different sizes held at constant density (Bouma et al. 1995). This study aimed to find evidence of R_0 being dependent on absolute population size. While population size is important in determining epidemic dynamics, it is insufficient to describe transmission rates from one individual to the next. The size of a population is important in determining the invasibility of a host population and its ability to support endemic disease (see Lloyd-Smith et al. 2005for review). The critical community size (CCS) of a population will depend upon R_0, but this does not mean that population sizes above this threshold should be expected to affect R_0. While conclusively demonstrating that transmission of PRV was independent of population size, this study does not address whether transmission was density dependent.

Studies manipulating livestock densities rather than numbers are more relevant to studying the influence of contact rates on disease transmission. Funk et al (2007) manipulate the stocking densities of pigs and examine the effect on shedding of *Salmonella enterica*. Experimental groups were classed as either high stock density of 31 pigs/pen (6.5 ft^2/pig or 0.154 pigs/ ft^2) or a low stock density of 25 pigs/pen (8.0 ft^2/pig or 0.125 pigs/ ft^2). It was found that pigs

stocked in higher density pens were not more likely to shed *S. enterica*. The high stock density was only 18% higher than the low. So although no evidence of density dependence was found, it is possible that the experimental manipulation on density was not severe enough to detect a significant change.

The example above highlights a potential problem with studies manipulating densities of livestock: livestock hosts are typically large, and implementing strong manipulations of host density with sufficient replicates to ensure statistical power may be unfeasible due to space, time or monetary constraints. Smaller animals may be housed at a wider range of densities within the strict controls of a laboratory setting.

Studies on laboratory populations of the Indian meal moth, *Plodia interpunctella*, have demonstrated one mechanism by which R_0 may be influenced by population density. Knell et al. (1996) show for infection by the bacterium *Bacillus thuringiensis* the transmission co-efficient, β, was best described by a linear function of host density, such that $\beta = \beta' + aN$. Where β' is an intercept and a is the gradient of β along host density N. Individuals infected with *B. thuringiensis* are infective upon death and transmission is by cannibalism of infectious cadavers. Further study of infection on the similarly transmitted *Plodia interpunctella* granulosis virus (PiGV) (Knell et al. 1998a; Knell et al. 1998b) found transmission to be explained by the same formulation of β. This has important implications for the effect of density on disease transmission. Previous density dependent models assume β is constant and the rate at which new infections occur to vary linearly as a function of the densities of infective and susceptible individuals and β (equation 2.1). By demonstrating β may be dynamic and not constant Knell et al. have shown that the force of infection, and thus R_0 does not necessarily vary linearly with host density. Formulation for R_0 of these systems therefore does not follow the standard density or frequency dependent formulations outlined in equations 2.3 and 2.4, rather it is given by:

$$R_0 = \frac{(\beta' + aN)N}{\mu + dN}$$ **Equation 2.6**

Where μ is the rate of decay of infectious cadavers and *d* is the rate of cannibalism (removal) by susceptible hosts. This relationship between R_0 and host density may be exponential when rates of cannibalism are low (as for *B. thuringiensis*) and asymptotic when they are high (as for PiGV). These studies certainly showed evidence of a type of density dependent transmission, although not in the "random mixing" sense used in traditional epidemiological models. They also demonstrate that R_0 may not vary linearly with host density. However transmission is through fomite rather than directly from an introduced infective individual. Dead hosts are simply an infectious object, and a food source. Increased density of infectious objects may simply represent an increase in this food source, which becomes less limited as more individuals succumb to infection.

Further support for non-linear dependencies between disease transmission and host density in laboratory populations is provided by Greer et al. (2008). In a study of transmission dynamics of *Ambystoma tigrinum* virus (ATV) infecting larval tiger salamander, *Ambystoma tigrinum nebulosum*, populations it is found that transmission was best modelled by a power, or negative binomial function. Traditional density dependent and frequency dependent models (equations 2.1 and 2.2) provided extremely poor fits to data. In striking similarity to PiGV transmission dynamics (Knell et al. 1998b) discussed above, it is found that transmission of ATV saturates at high population densities. Unlike PiGV dynamics, the transmission of ATV is through direct contact, not through cannibalism of infectious cadavers. ATV is therefore a directly transmitted disease and not fomite transmitted, as is the case for PiGV. Greer et al. demonstrated transmission can scale non-linearly with host density for a directly transmitted disease.

In experimental studies population density can be strictly controlled, and with some species the assumptions of spatial homogeneity and random mixing are more likely to be upheld on smaller laboratory scales in animals that are limited in their social behaviour. But even when these assumptions seem reasonable, the relationship between R_0 and population density cannot be expected to follow a linear function, which questions the adequacy of traditional density dependent modelling.

2.6 Evidence of density dependent transmission from naturally occurring epidemics

Tracking the spread of a disease in a wild population is a much less precise process than using a contained study population. Wild populations produce offspring, and so there is an (often unknown) influx of susceptible individuals. They may exhibit behaviours that affect disease transmission either positively or negatively such as territoriality, sociality, or group living. These behaviours may change seasonally. Seasonal changes may also indirectly affect disease transmission, for example increased animal densities may occur around water holes in dry seasons. In such incidences host populations may be structured more similarly to that shown in Figure 2.2 than Figure 2.1; where transmission at local scales between individuals within clustered groups is more important than transmission throughout the entire range of a population. This would implicate local density as being responsible for driving density dependent transmission rates, and not global density.

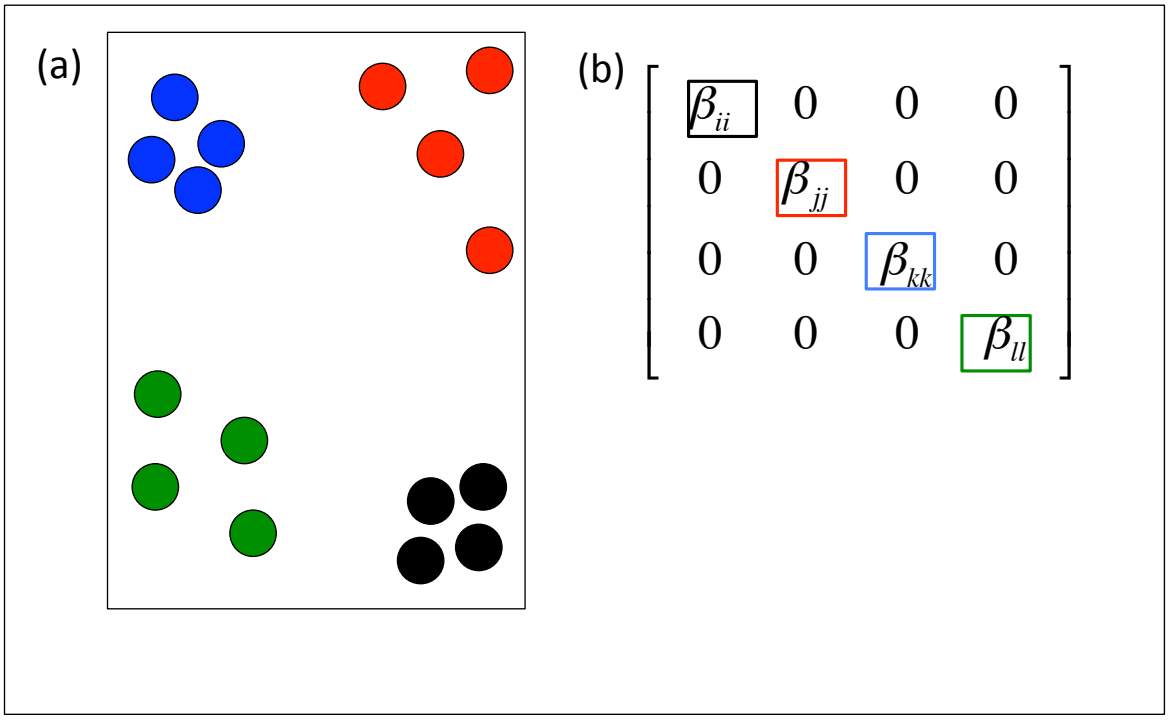

Figure 2.2 Schematic of the effects of heterogeneous population structure on the basic reproductive number. The global density of the host population (a) is equal to that of Figure 2.1(a). Groupings of individuals (represented by different colours), may vary in their local densities. If transmission only occurs within groups, and transmission between individuals within a group is equal, matrix (b) describes the within group transmission rates which may vary as a function of that groups density.

By examining epidemics in humans many of the aforementioned difficulties can be negated. Studies in human epidemiology were the original source of the "cities and villages paradigm" (Anderson & May 1991b; May & Anderson 1984), which predicts the average force of infection will be concentrated in heavily populated urban dwellings. This hypothesis has been explored widely through examination of measles epidemics. Despite this exploration the propensity of density and frequency dependence is still blurred. In a study into the use of vaccination to control infectious disease, Anderson and May (1982) note variation in measles' R_0 between cities and villages (range between 14 - 18), and predict that the necessary vaccination coverage to eliminate the disease should be lower in rural than urban environments. The implications of this observation are dependent on whether rural settlements have lower densities, or just lower numbers of susceptible hosts. However, this theory assumes complete separation between urban and rural settlements, akin to the dynamics illustrated in Figure 2.2, when in reality individuals occupying these environments are inherently linked. There is movement, and thus contact, between these populations. Grenfell and Bolker (1998) examine the metapopulation dynamics of measles epidemics, and identify a "hierarchy of infection" whereby large epidemics originate in large urban populations and diffuse to smaller rural populations. These epidemics begin regularly and predictably in larger urban habitats, but the spread to smaller rural habitats is dependent on stochastic processes, making transmission to rural habitats harder to predict (Finkenstadt et al. 1998). Whilst this work supports the "cities and villages paradigm", it again fails to address the key issue of local density in human settlements: measles is predominantly a childhood infection, and cyclical outbreaks coincide with the school year (Bjørnstad et al. 2002). This implies that schools could be taken as the local unit on which density should be measured. Rather than comparing urban and rural dwellings, it would be more useful to examine if schools with different densities of children varied in their measles dynamics. On this basis these studies cannot inform of the relationship between host density and measles transmission.

In contrast to Anderson and May (1982) Bjørnstad et al. (2002) found no evidence of R_0 varying between cities and villages when analysing pre-vaccination era data on measles in England and Wales (mean R_0=29.9, S.E.= 0.9). The value of R_0

estimated is higher than those estimated by Anderson and May (1982), and this is attributed to the inclusion of seasonal forcing in Bjørnstad et al's. (2002) modelling of the data. Bjørnstad et al also demonstrate that the seasonality in measles epidemics coincided with school terms, with higher rates of transmission during term time than during school holidays. Thus the seasonality of measles epidemics could be explained by periods when there were high-density patches of children. Despite reaching the conclusion that measles demonstrates frequency dependent transmission, Bjørnstad et al. (2002) acknowledge that local density may be important in explaining the transmission dynamics of measles; and that this may conceivably be the same between city and village schools. This would suggest a combination of density and frequency dependent transmission may best describe measles outbreaks with density dependent dynamics within schools and frequency dependent dynamics between schools akin to the dynamics described by Figure 2.3.

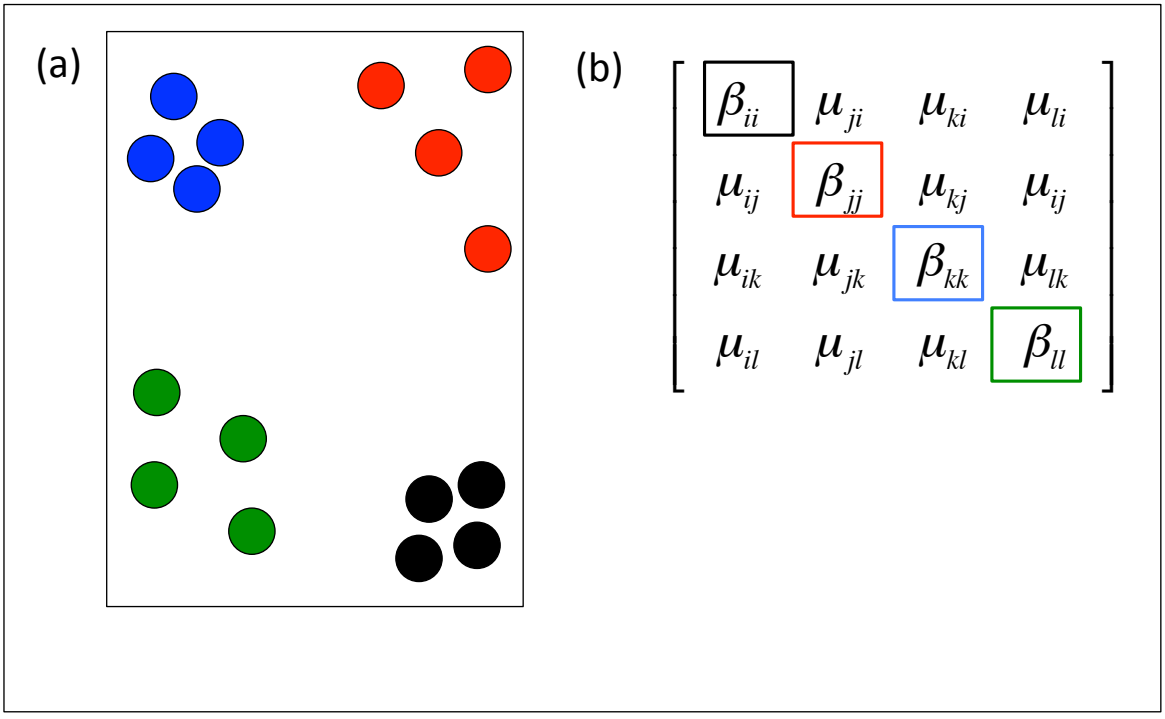

Figure 2.3 Schematic of the effects of heterogeneous host density and allowing contacts between groups on the basic reproductive number. The global density of the host population (a) is equal to that of Figure 2.1(a). Groupings of individuals (represented by different colours), may vary in their local densities. If transmission is density dependent within groups, and transmission between individuals within a group is equal, and transmission between groups is frequency dependent then transmission can be described by matrix (b). μ terms describe the frequency dependent transmission rates between groups. Each row of this matrix may have its own local density, N_i *attributed*.

The examples discussed above only cover a fraction of the work on measles that has influenced modern epidemiological theory concerning density and frequency dependent transmission. In retrospect, a pathogen that exhibits such marked seasonality, and age preference for hosts makes quantifying R_0 and the effective densities of hosts extremely difficult. Measles has undoubtedly been used as a "model pathogen" for studying transmission dynamics as incidence has been recorded over many years, so data is plentiful. However the analysis of these data, although informing many aspects of theoretical and practical epidemiology, does not provide convincing evidence of being exclusively density or frequency dependently transmitted. It does however highlight the complex relationship between host behaviour and ecology with transmission, and supports the notion that density and frequency dependence are two ends of a continuum (McCallum et al. 2001), and measles may lie somewhere between the two.

One of the single biggest challenges in studying epidemics is tracing infection throughout a population. In a study examining the feasibility of rabies eradication, Hampson et al. (2009) traced the history of infectious contacts to study the biting behaviour of rabid dogs. Through quantifying biting behaviour and the probability of infection given a successful bite ($\boldsymbol{P}_{(\text{rabies}\,|\text{bite})}$) R_0 could be calculated. Comparison of two dog populations with different densities (Serengeti, 9.38 dogs/km^2; Ngorongoro, 1.36 dogs/km^2) showed no significant differences in R_0. Furthermore, when values of R_0 obtained were compared with estimates from previous epidemics occurring worldwide no conspicuous differences were observed. The transmission dynamics of rabies were insensitive to the density of available hosts, but are affected by demographic turn-over (Hampson et al. 2009; Lembo et al. 2010). This study questions the previous assumptions of density dependence on which control measures have been based on (Coleman & Dye 1996; Sterner & Smith 2006). This further illustrates the need for empirical evidence on transmission dynamics, as assumptions based on the theories of density and frequency dependence may be too simplistic to reflect reality.

Rabies is transmitted during an uncommon and memorable event that can be retrospectively recorded. The transmission events of most diseases are less easily identified. This results in a situation where all that can be measured is the prevalence of a disease in a population at one or more points in time. This can

present problems in observing rates of infection. Brown and Brown (2004) examined the prevalence of the parasitic bug, *Oeciacus vicarious*, in colonies of the cliff swallow, *Petrochelidon pyrrhonota*, by fumigating nest sites weekly and collecting dead bugs from nests. This allows rate of infection to be quantified as bugs/nest/week. It was found that nests in larger colonies of cliff swallow acquire parasitic bugs at faster rates. As only colony size was measured, and not density this alone cannot be considered as indicative of density dependent transmission unless we assume colony size and colony density are positively correlated. However, in a previous study by the authors (Brown & Brown 1996) it was found that the spatial organization of nests influenced the dispersal of bugs: rate of inter-nest movement of bugs is negatively correlated with distance to the nearest neighbouring nest. Nests are stationary fomites, and this phenomenon parallels what is observed with separation distances in plant hosts. This would be suggestive of a form of density dependent transmission as separation distance is inversely related to density (equation 2.5). However, Brown and Brown (2004) claim similar densities of nests at all sites although no formal measurement or methodology is provided. Instead higher rates of nest colonisation by bugs is explained by large colonies having more transient individuals that travel between colonies, picking up and spreading the infection. If so, metapopulation dynamics may be influenced by density of colonies (rather than density within colonies), which may prove important for disease transmission. However, if movement between colonies is based on neighbour quality rather than neighbour proximity (suggested by Brown & Rannala 1995) then transmission on this scale would be better characterised by frequency dependence, as demonstrated in Figure 2.3. In reality, the body of work on this system is lacking the necessary information on within and between colony densities necessary for reaching a firm conclusion about transmission dynamics. It would appear, preliminarily, that transmission between nests (within-colony transmission) might be a density dependent process; while transmission between colonies tends towards the frequency dependent end of the spectrum.

Transmission rates of disease in natural populations are governed by more than simply host density. Not all individuals contribute equally to the spread of disease, and the above examples demonstrate that population demography, sociality and dispersal may regulate disease transmission more strongly than

densities of available hosts. To better understand the impact of density on rates of disease transmission it will be necessary to examine density at smaller scales, and consider the impacts of heterogeneity in population density at larger scales. By applying models which accounted for population age-structure and heterogeneous population mixing to data from a phocine distemper virus (PDV), outbreak Klepac et al. (2009) demonstrate the importance of heterogeneous density dependent dynamics in a host colony. When the entire colony was modelled as experiencing equal transmission rates, density dependent transmission best explained the observed epidemic progression. However, models incorporating the age and social structure of the colony showed that less social juveniles and adults exhibited frequency dependent transmission, and only the highly sociable, and aggregated sub-adults showed strong evidence of density dependent dynamics. Transmission between colonies is frequency dependent (Swinton et al. 1998), which is again indicative that metapopulation dynamics would be useful in explaining the spread of disease at larger scales. Figure 2.3 thus broadly explains these dynamics. However the values of β in the transmission matrix (Figure 2.3(b)) may be an over-simplification, and would be better represented as an age structured transmission matrix. This example illustrates that host variation may cause heterogeneous disease dynamics at local scales, with only some cohorts exhibiting transmission rates affected by density. Again, density dependence is of most importance at local scales within a colony and specifically within a single age class.

The impact of host density on transmission rates in naturally occurring epidemics is certainly more complex than in experimental set-ups. Outwith the controlled conditions of a laboratory host ecology, behaviour and demography may affect transmission rates more strongly than, or in conjunction with population density. The assumption that transmission rates increase with density due to random assortment of hosts in space is too simplistic; and its real world applicability is questionable. More work is required to understand how variation in population densities in nature reflects changes in population and disease dynamics.

2.7 Insights from transmission networks

Network models provide the most powerful insights into heterogeneity in disease transmission, being able to demonstrate the impacts of differing contact

probabilities between individuals in a population, or between groups in a metapopulation. This differs greatly from compartmental models as the as there no need to make the assumption that every individual in the host population is "connected" to every other individual in the population, and the strengths of connections between individuals may vary. Network theory predicts that increasing heterogeneity in the potential number of infectious contacts between individuals will lead to an increased R_0, such that even when mean transmission rates are low, high levels of heterogeneity can lead to large disease outbreaks (Kiss et al. 2006; Porphyre et al. 2008). One potential cause of this heterogeneity could be heterogeneity in the density of a host population. This would be the case if the probability of infectious contacts between network nodes (which may defined as individual hosts, or groups of hosts) were proportional to the physical distance between them – as distance between nodes would decrease as density of nodes increased (Equation 2.5). In the case of "small-world networks", where most nodes are connected by relatively few steps through intermediate nodes, the effect of spatial clustering is predicted to increase the spread of disease (Aparicio & Pascual 2007; Dobson 2004). Calculating R_0 from transmission networks is more complicated than the traditional S-I-R models proposed by Anderson and May (1982). Arguably the simplest form of network model is the "who acquires infection from whom matrix" (WAIFM), where R_0 can be calculated as the dominant eigenvalue of this matrix (Anderson & May 1991b). This matrix takes the form:

$$W = \begin{bmatrix} \tau_{1,1} & \tau_{2,1} & \cdots & \tau_{n,1} \\ \tau_{1,2} & \tau_{2,2} & \cdots & \tau_{n,2} \\ \vdots & \vdots & \ddots & \vdots \\ \tau_{1,n} & \tau_{2,n} & \cdots & \tau_{n,n} \end{bmatrix} \qquad \textbf{Equation 2.7}$$

Where the elements in this matrix specify the rate of infection between all possible combinations of individuals considered by the model. This matrix need not be symmetrical: meaning that some individuals may be more infectious, or more susceptible than others. Should the average rate of infection in this matrix increase with host density, so too will the dominant eigenvalue, and thus R_0.

In practice, tracing all contacts between individuals in wild populations is problematic. The degree of "connectedness" between individuals may however

be inferred from standard ecological field techniques such as radio-tracking and capture-mark-recapture methods. Perkins et al (2009) utilise exactly these two approaches to derive social networks for rodent populations and then build transmission networks for hypothetical parasites. They find that when these two methods were undertaken simultaneously that radio-tracking produced more informative networks when rodent density was low and capture-mark-recapture produced more informative networks when rodent density was low. Both contact networks were well described by negative binomial distributions implying that regardless of density a small proportion of individuals were responsible for a disproportionately high number of contacts with the rest of the population. This is a similar result to that found by Hampson et al (2009), where a small proportion of domestic dog populations were responsible for a large number of infective contacts spreading rabies. However, unlike the study of rabies in domestic dogs by Hampson et al (2009), Perkins et al (2009) find that mean contact rates were higher when population density was high. However, they also note increased levels of super-spreading individuals (those with disproportionately high contact rates) when population density was low. This is likely attributable to animal behaviour, where in low density populations animals may have more freedom to forage more widely, or move further to utilise unoccupied habitats.

As a single summary statistic, R_0 poorly distinguished between these two increased modes of transmission. Instead, measures of *closeness* and *betweenness* were more applicable to describing changes in the contact structure of the population: where *Closeness* provides an index of the extent to which an individual is in the middle of a given network, where at its maximum a single individual is connected to all others in the population, acting as a central "hub" of connections, and at its minimum all individuals are equally well connected (homogenous distribution); in comparison, *betweenness* is a measure of the number of paths that pass through a single individual along the shortest path between all other individuals and is, conceptually, a measure of the flow of a pathogen through a network. It is found that betweenness is positively correlated with rodent density, suggesting faster spread of infection in high density populations even if R_0 does not differ significantly in high and low density populations. It is therefore clear that host density has the potential to

influence epidemic dynamics, and similar R_0 values between populations of differing densities may not be indicative of a lack of density dependent transmission as this may be masked by heterogeneity in population contact structures.

2.8 Conclusions

The relationship between host population density and disease transmission is complex and likely variable dependent on the host-pathogen system in question. The mechanism behind any relationship between population density and contact rate is variable between species due to differing ecologies, and individuals due to behavioural and social differences. Contact between individuals does not increase due to "random mixing" of populations. Types of contact, such as those resulting from sexual, aggressive or grooming behaviour will be affected differently by increases in density. There is no *a priori* reason to assume a linear increase in contact rate with density due to random mixing of populations. In reality, this may not be a useful way to think of this relationship: It will not aid in identifying causes of increased disease spread, or reasons for heterogeneity in contact rate which may be important in determining heterogeneity in the spreading of disease. Heterogeneity in disease spread may even mask the effects of host population density on disease transmission when examined under these assumptions.

Controlled experiments manipulating host density further re-iterate this conclusion and demonstrate that host density may relate non-linearly to R_0, and different pathogen-host systems may have different formulations of a density dependent R_0. The simple, but commonly used, formulation of R_0 shown by equations 2.3 demonstrates an expected linear dependency of R_0 on host density, N. Again, there is no reason to assume this is the case. Density dependent transmission can be demonstrated under experimental conditions, but is better explained when the static parameter β is replaced by a dynamic function or when exponential functions are used to relate density to contact rates. This leads to R_0 values that vary non-linearly with the density of the host population. From such studies it is shown that R_0 may have an asymptotic relationship with host density, suggesting saturation in transmission rates at high densities may be expected.

In naturally occurring epidemics density dependence is harder to demonstrate. The mechanisms behind which increased density operates to increase contact rates are complex, and dependent on ecology, behaviour, sociality, seasonality and demography. Given this, the simplistic theories of density and frequency dependent transmission will likely prove inadequate for studying host-pathogen interactions responsible for variability in transmission. As stated previously, these two types of transmission should be thought of as two ends of a continuum. Where a pathogen lies on this continuum will not only be dependent on the pathogen and the host population. It also depends on the scale at which the system is examined. The density of groups within a meta-population can influence disease spread through different mechanisms than the local density within a single group. While density on larger scales ultimately affects transmission dynamics and disease persistence, it would be sensible to consider local density dependence in calculations of R_0.

Empirical studies reveal the complexity and variability of the interaction between host density and R_0 highlighting the inadequacies of the commonly assumed linear relationship between host density and pathogen transmission. It is often considered within the theorist's remit to provide models with "general applicability". It is accepted that these general models are always, to some extent, wrong. There is a balance to formulating models to maximise generality while ensuring they are not "importantly wrong" and therefore useful. The formulation of R_0 for density dependently transmitted pathogens is derived from an extremely general model with some unrealistic prior assumptions. There is no doubt that traditional epidemiological models have been useful, but when confronted with data from the real world these models appear inadequate. The assumptions of homogeneous mixing, random movement and a linear relationship between density and transmission rate prove "importantly wrong" when it comes to explaining transmission in specific host-pathogen systems. Where density dependent transmission is exhibited, formulations of R_0 that relax the assumptions of homogenous hosts and linear dependencies on density have proven successful and have the potential to improve disease management strategies and better our understanding of disease ecology, and when contacts between individuals are explicitly modelled in networks it can be seen that changes in population density may impact on

3 Exploring a disease community in a British population of rural red foxes

3.1 Introduction

3.1.1 The concept of pathogen community

Individual animals are subject to infection by a plethora of pathogens, including – but not limited to – viruses, bacteria, protozoa and helminthes. Infection by these agents may occur sequentially and/or concurrently (Petney & Andrews 1998). Pathogens exist in communities. These communities can be considered as the assemblage of pathogen species infecting a single host, commonly defined as the *infracommunity*; the number of pathogen species present in a population of hosts, defined as the *component community*; or the community of pathogens infecting a community of hosts – the *compound community* (Bush & Holmes 1986).

The concept of interactions between pathogens was recognised by Petney and Andrews (1998). They noted in their review the need to combine aspects of parasitology and microbiology in an epidemiological framework in order to determine the effects of interactions between pathogens on host populations. There is much debate in the literature as to how important these interactions are likely to be (Cox 2001; Graham et al. 2007; Harbison et al. 2008; Pedersen & Fenton 2007). It is important to note that just because two infectious agents concurrently occupy a host, it does not mean they are necessarily competing, or interacting in any other way. Interactions between different pathogens may be subtle, and infrequent. It is not the case that we should expect all concomitant infections, or infracommunities of pathogens to have effects significantly different from single infections within a host population. There is however little doubt that specific combinations of pathogens do interact with each other (Balestrieri et al. 2006; Cox 2001; Dobson & Barnes 1995; Pedersen & Fenton 2007). These interactions may be competitive in nature – limiting the size of populations (the pathogen burden of individual hosts) within an infracommunity (Heins et al. 2002; Holmes 1961; Read 1951); they may be facilitative, affecting

the susceptibility and infectiousness of the host (Graham et al. 2007), which would be expected to affect the component and compound communities. The first step towards detecting such interactions is to look for pathogens that occur together more or less frequently than expected by random occurrence in a population.

3.1.2 The red fox as a host population

We look for evidence of interactions in a pathogen community infecting a population of red foxes, *Vulpes vulpes*. This small to medium sized canid has proven highly adaptable, and has established large urban populations across Europe, most notably in the UK, where the "urban fox phenomenon" has been observed and reported since the 1930s (Harris & Rayner 1986). Like most canines, foxes have a varied diet. Unspecialised scavenging and predation are however the two primary foraging techniques utilised by this opportunistic omnivore (MacDonald & Reynolds 2004). Wide ranging diet and scavenging put foxes at risk for infection with large numbers of helminth and protozoan parasites, many of which are zoonotic or of threat to livestock and companion animals (Simpson 2002). Foxes are territorial and form plastic social groups (White & Harris 1994). Territory size is variable, with a high degree of overlap, often leading to aggressive encounters on territory borders (White & Harris 1994), which may increase seasonally with, for example, breeding season or following outbreaks of disease (Baker et al. 2000; Doncaster & Macdonald 1991). This behaviour and plastic sociality may favour the spread of directly transmitted disease.

The hosts studied here are from a red fox population in rural Kent, UK. British red foxes exist at higher densities than elsewhere in Europe (Webbon et al. 2004). This may be attributed to a long period of freedom from terrestrial rabies (Macdonald et al. 1981) and the comparatively low levels of population control employed in the UK. This allows foxes in the UK to maintain larger population sizes than elsewhere in Europe with total numbers of around 258 000 (Webbon et al. 2004) Diseases infecting fox populations in the UK, specifically England are well documented (see Simpson 2002; Smith et al. 2003), with many infections being the subject of economic or public health concern, affecting humans, companion animals and livestock.

3.1.3 Pathogens of interest

We consider protozoa, viruses, bacteria, helminthes and mites as some of the constituents in the pathogen community of foxes. Specifically:

The obligate intracellular parasite *Toxoplasma gondii* is capable of infecting almost all warm-blooded animals, but the definitive hosts are cats (Murphy et al. 2008). Intermediate and incidental hosts (including humans, livestock and foxes) are infected through one of three routes: horizontally through ingestion of contaminated food or water with infectious oocysts excreted by an infected cat; consumption of undercooked meat containing tissue cysts; or vertically from mother to foetus across the placenta. Although foxes are essentially a dead end host for this disease (incapable of shedding the parasite into the environment, and are not preyed upon by any feline species) they provide an excellent indicator for levels of *T.gondii* in a given ecosystem, thus acting as a sentinel species (Murphy et al. 2007). This parasite also has the potential to interact with other pathogens within a host. Its chronic persistence elicits and maintains a Th1 immune response, limiting the ability of the host to mount a Th2 response against subsequent infections (Graham 2002).

Canine adenovirus-1 (CAV-I) and canine adenovirus-2 (CAV-II) respectively cause infectious canine hepatitis (ICH) and infectious canine laryngo-tracheitis in wild and domestic canines (Chaturvedi et al. 2008). This disease has been shown as a cause of mortality in other red fox populations (Woods 2001) and has been found to have high prevalence in carnivore species worldwide including the endangered island fox, *Urocyon littoralis*, and giant panda, *Ailuropoda melanoleuca* (Clifford et al. 2006; Qin et al. 2007). CAV-I is shed by infected animals through their urine and faeces. CAV-II is spread as an aerosol and is quickly inactivated in the environment, whereas CAV-I has the potential to survive outwith a host for several months. These viruses can potentially have impacts on the demography of red fox populations, causing increased mortality rates; primarily of juveniles (Clifford et al. 2006; Woods 2001).

Canine distemper virus (CDV) is a highly infectious directly transmitted viral disease, capable of infecting a wide range of wild and domestic carnivores (Appel 1987). While known to be endemic in mainland Europe (Gortazar et al.

2007) there is surprisingly little reported on the prevalence of this disease in the UK. A previous effort to detect the disease in wild badger, *Meles meles*, populations in England found a complete absence of the disease (Delahay & Frolich 2000).

Angiostrongylus vasorum is a metastrongyloid nematode that parasitises canids as the definitive host. Infection is via an intermediate (gastropod) or paratenic (for example amphibian) host (Traversa et al. 2008). The adult stage parasite resides in the right ventricle and pulmonary arteries of the definitive host, causing severe respiratory and circulatory distress, usually resulting in death (Morgan et al. 2008). *A. vasorum* was previously thought to be restricted to the South-West of Britain, but anecdotal evidence since the late 1990s suggests a northward spread of the disease (Morgan et al. 2008), with cases now reported as far north as Scotland (Helm et al. 2009).

Sarcoptic mange is caused by the parasite *Sarcoptes scabei*. The parasite is highly nonspecific and highly pathogenic transferring readily between sympatric species (Newman et al. 2002) either through direct contact, or indirectly from the environment. All stages of the parasite life cycle are capable of moving to the surface of the host's skin, and all are infectious (Soulsbury et al. 2007). The first major mange epizootic in Britain to be recorded was in spring, 1994 in Bristol (Baker et al. 2000), leading to a 90% decline in the fox population. In this case the disease almost certainly did not originate in Bristol, mange has been enzootic in Britain since at least the 1940s (Soulsbury et al. 2007), and the reasons as to why this particular epidemic was so severe remain unclear.

Leptospirosis is a worldwide zoonosis, caused by bacteria within the genus *Leptospira interrogans*, which consists of over 250 pathogenic serovars. Infection is acquired orally, either from the environment or through consumption of infected prey items, and shed back into the environment through the urine of the infected individual (Diesch et al. 1976). Leptospirosis in the UK is grossly understudied. Serovars *L. canicola* and *L. icterohaemorrhagiae* are routinely vaccinated against in domestic dogs, as pre 1960 these serovars were the most commonly observed causes of canine leptospirosis (Geisen et al. 2007). The main reservoir for each of these serovars is considered to be wild rodents, and both have been shown to infect foxes in Croatia (Milas et al. 2006). There have been

no wildlife vaccination programs against these serovars in the UK. As such it may be assumed that rodents still act as a reservoir for this disease. Although it should not be assumed that the most common serovars present in the UK have remained the same since the 1970s. Under these assumptions it is expected that rural foxes will be exposed to this disease, as rodents form an important component of their diets.

The work presented here utilises direct testing for the presence of pathogens as well as an examination of pathologies present at the animals time of death which may be indicative of disease which was not directly tested for. Although the pathological diagnoses provide a less conclusive diagnosis of the infectious agent responsible, it allows us to consider a wide range of pathogens that may be present within the host population. We aim to present the prevalence of disease found in the animals collected and examine patterns of co-infection in the host population studied. We shall then examine if the condition of individual hosts is correlated to the likelihood of infection with a range of diseases.

3.2 Methods

3.2.1 Sourcing of fox cadavers

Foxes were provided by a private estate in Canterbury, Kent, UK (51°13'20.68"N, 0°59'11.24"E). The site is host to ground nesting birds such as lapwings, *Vanellus vanellus*, and oystercatchers, *Haematopus ostralegus*. Foxes are considered by estate managers to pose considerable threat to ground-nesting birds such as these, and so populations are controlled. The foxes used here were shot between May 2009 and September 2010 and stored at -30°C before transport back to the Glasgow School of Veterinary Medicine for post-mortem. Foxes were aged based on incisor wear (Harris 1978) and classed as pups (<1 year), yearlings (>1 year, <2 years) or adults (>2 years).

3.2.2 Sample collection and analysis

Post-mortems were carried out at the University of Glasgow School of Veterinary Medicine pathology laboratories. Cadavers were weighed, sexed, aged and scored on the standard canine body condition scale from 1 -5 (table 3.1). Post-mortem examination began with an external examination of the skin; specifically

examining the carcass for visible skin pruritus consistent with ecto-parasite infection. Foxes were neither collected nor stored in isolation from each other making cross-contamination between cadavers likely. As such skin scrapings were not taken from cadavers, as we could not guarantee an increased incidence of false positive results. Cadavers are thus reported as being positive or negative for general skin disease based on these criteria.

Score	Criteria
1	Ribs, spine and pelvis easily visible. Loss of muscle mass. Very obvious waist.
2	Ribs, spine and pelvis visible. Waist is obvious.
3	Ribs, spine and pelvis easily palpable, but not visible. Waist is obvious.
4	Ribs, spine and pelvis are not visible and hardly palpable. Waist is absent and there are heavy abdominal fat deposits.
5	Ribs, spine and pelvis not visible and not palpable. Waist is absent. Massive fat deposits on the chest, spine and abdomen. Abdomen is distended.

Table 3.1 Criteria used to determine the condition score of fox cadavers.

Samples collected from the cadaver required opening the body cavity. This was accomplished with a longitudinal cut on the ventral surface of the abdomen. Serum samples were obtained from the heart ventricles where possible, with one sample stored in a plain collection tube and another in an EDTA collection tube (BD Vacutainer® blood collection tubes). Following this the lungs were examined and sampled. The lungs were first examined for scarring and signs of inflammation that may have been indicative of parasitic infection. If present, tissue samples were taken from these areas, with one sample stored frozen and another stored in formalin. Finally a whole kidney was removed from each

cadaver and stored in formalin. The usage of each of these samples is given in table 3.2.

Sample	Storage	Sample tested for:	Method
Serum	Plain tube	CAV-I, CAV-II, CDV	PCR
Serum	EDTA tube	*Toxoplasma gondii*	Latex agglutination test
Lung	Formalin	Bacterial and parasitic aetiologies	Histo-pathology
Kidney	Formalin	Bacterial aetiologies	Histo-pathology

Table 3.2 Summary of the collection and usage of samples collected during post-mortem examination of foxes.

Samples were submitted to the University of Glasgow veterinary diagnostic service for testing. PCR analysis for CAV-I, CAV-II and CDV returns simply a positive or negative result for each sample. The latex agglutination test used for *T. gondii* returns an antibody titre. We take a titre of ≥1:32 to be indicative of a positive infection, as used for studies on stray dogs (Ali et al. 2003; Tsai et al. 2008). Lung and Kidney samples were submitted to the University of Glasgow veterinary diagnostic service for histo-pathology. These samples were examined for aetiologies characteristic of parasitic and bacterial infections. Histo-pathological methods cannot be expected to diagnose the exact pathological agent responsible for the observed disease in an animal, but allows us to consider a wider range of pathogens that may be responsible for a presented pathology. For data analysis we thus consider animals testing either positive or negative for general kidney and or lung disease.

3.2.3 Data analysis

As cadavers were collected from a population where total size was not monitored calculating prevalence thresholds for the detection of disease cannot

be calculated exactly. A conservative approach will thus be taken to calculate these thresholds. The most recent population estimate (1999-2000) of foxes in this S.W. England was 34,399, at a mean density of 0.79 foxes km^{-2} [95% CI: 0.46 -1.12] (Webbon et al. 2004). As foxes are not restricted to the area where shooting took place, the area sampled is difficult to quantify. An approximation of the population sampled must therefore be made. Assuming all foxes were shot at approximately the same location, the area foxes were sampled from is given by a circle where the radius is the maximum distance a fox is likely to travel. We take the maximum distance a British rural fox is likely to travel as 52 Km (Trewhella et al. 1988). This approximation assumes that all foxes will travel this maximum distance and that foxes have an equal probability of being sampled regardless of how far away they are from the centre of the circle. These assumptions are clearly un-realistic, and will likely lead to an over-estimation of the population size from which samples were obtained. This is predicted to give an extremely conservative estimate of detection levels. The probability of detecting a disease present in a population ($\mathbf{P}_{Detection}$) depends on the population size (N), the number of infected individuals (M) and the sample size (n). $\mathbf{P}_{Detection}$ is defined as one minus the cumulative probability that a sampled individual is uninfected with a disease and is calculated with equation 3.1.

$$\mathrm{P}_{Detection} = 1 - \prod_{i=0}^{n-1}\left(\frac{N-M-i}{N}\right) \qquad \textbf{Equation 3.1}$$

Statistical analyses were performed in R v2.14.0 (The R foundation for statistical computing; http://www.R-project.org). To analyse co-infection relationships we test whether any of the diseases here occur together more frequently than expected by chance alone using contingency tables. Fisher's exact test was used to help account for the small numbers of positives within these data. The same approach was taken to test if any of the diseases tested for occurred in combination less frequently than expected by chance alone. Odds ratios and associated 95% CI were calculated manually, and sample size calculations were performed using apriori tests in G*Power v3.1.3.

Each disease was used as a binary response variable in generalised linear models (GLMs) with binomial error structures to establish if the probability of an

individual testing positive for an infection could be explained by the age or gender of that individual, with an interaction term included.

The effect that multiple infections have on host condition was also examined. As all individuals fell within one of two condition scores it was modelled using GLMs with binomial error structures. Each disease, host age and gender were included as explanatory variables with all possible interaction terms.

General linear models were used to test if disease accounted for variation in the observed weights of the cadavers. Each disease was included as an explanatory variable with all interaction terms included. Age and gender were also included as explanatory variables, with their interaction terms, in this model to control for natural variation between these categories.

All models described above are the full models used. Stepwise removal of non-significant variables was employed and optimum models were chosen based on AIC values.

3.3 Results

3.3.1 Disease Prevalence

Not all of the diseases tested for were present in this population sample. None of the foxes sampled tested positive for CDV or CAV-II. The minimum prevalence a disease can exist at allowing 95% probability of detection is 7% with a sample size of 42, as shown by Figure 3.1. These pathogens are therefore assumed to exist at prevalences of <7% and ≥0%. CDV and CAV-II were subsequently excluded from all analyses. Table 3.3 shows the prevalence for all pathogens and pathologies tested for. Sample size is variable as the samples obtained from some cadavers were too badly lysed for analysis. Figure 3.1 shows that the disease prevalence at which the 95% detection probability threshold occurs differs marginally between lowest and highest sample sizes obtained. With the 95% threshold for detection probability occurring at a prevalence 0.5% higher in the lowest sample size (n=39) than compared to the highest (n=42).

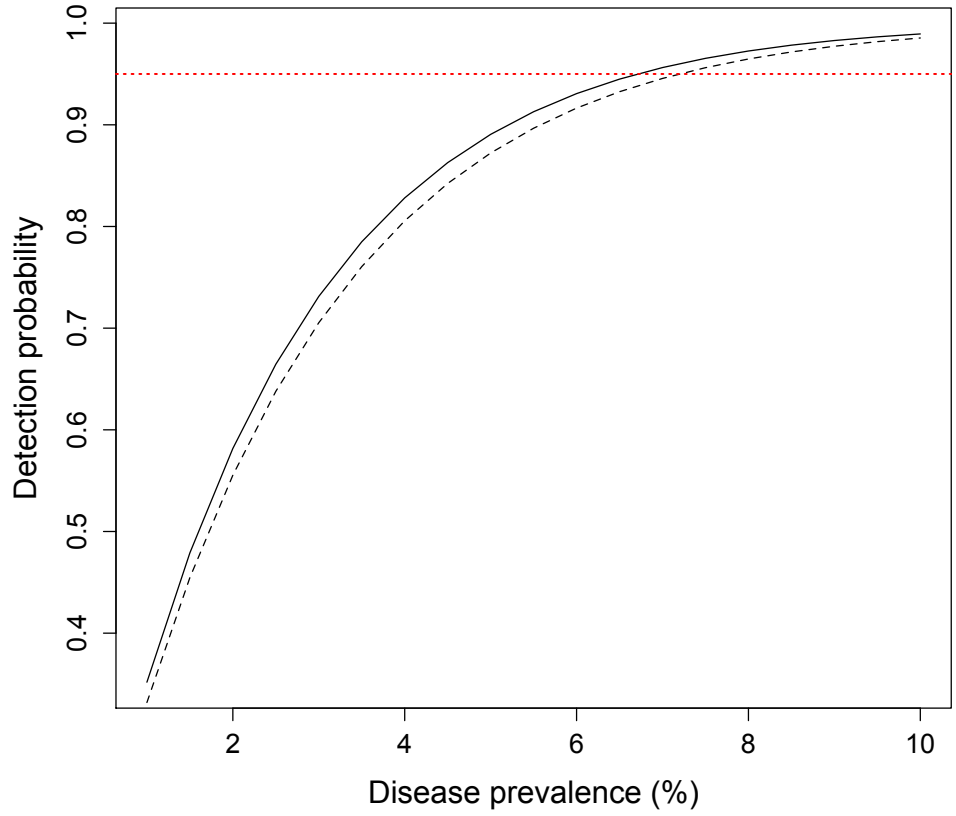

Figure 3.1 Detection probabilities of disease across a range of prevalences. Black lines represent detection probabilities for the highest and lowest sample sizes obtained (solid, *n*=42; broken *n*=39). Dotted red line illustrates the 95% probability detection threshold.

Of the specific pathogens tested for, *T. gondii* and CAV-I were present in the animals sampled at prevalences of 12.82% and 16.67% respectively. A much higher percentage of the population showed general pathologies that could be attributed to a range of pathogens.

Probability of detecting infection with *T. gondii* or CAV-I were not correlated with host age (p=0.713 and p=0.264 respectively), suggesting the likelihood of a fox acquiring infection does not increase with the length of time it has survived. There was also no significant difference in the probabilities of detecting infection with *T. gondii* or CAV-I between genders (p=0.086 and p=0.918 respectively).

Age had no significant effect on the probability of observing pathologies of the skin and kidneys (p=0.165 and p=0.843) but did have an effect on the probability of observing pathologies of the lung, with yearlings having the highest

probability of exhibiting lung pathologies (p=0.008). The probability of observing skin, kidney or lung pathologies was not dependent on gender of the fox being examined (p=0.462, p=0.315, and p=0.299 respectively).

Disease	Prevalence (%)	Sample Size
Toxoplasma gondii	12.82	39
CAV-I	16.67	42
CAV-II	0.00	42
CDV	0.00	42
Skin pruritus	9.52	42
Lung pathology	25.00	40
(parasitic aetiology)	*(10.00)*	
(parasitic and/or bacterial aetiology)	*(15.00)*	
Kidney pathology	45.00	40
(Chronic bacterial aetiology)	*(45.00)*	

Table 3.3 Disease prevalence in host population.

3.3.2 Co-infections

Despite 38% of foxes testing positive for more than one pathogen or pathological symptom only one combination occurs more frequently than expected by chance (Figure 3.2); this was the combination of the intra-cellular parasite *T. gondii* and the virus CAV-I (p=0.019).

	T.gondii	CAV.I	Skin	Kidney	Lung
T.gondii		*p= 0.02* OR= 7.5 up= 134.6 low= 0.4 n_{min}= 78	*p= 1* OR= 0 up= NaN low= 0 n_{min}= 374	*p= 0.156* OR= 4.4 up= 74.7 low= 0.3 n_{min}= 147	*p= 1* OR= 1 up= 12.3 low= 0.08 n_{min}>10000
CAV.I	✓		*p= 1* OR= 0 up= NaN low= 0 n_{min}= 227	*p= 0.22* OR= 2.8 up= 26.7 low= 0.3 n_{min}= 179	*p= 0.41* OR= 2 up= 17.2 low= 0.2 n_{min}= 450
Skin	✗	✗		*p= 0.61* OR= 0.4 up= 7.9 low= 0.01 n_{min}=456	*p= 0.63* OR= 0.5 up= 4.9 low= 0.04 n_{min}= 970
Kidney	✗	✗	✗		*p=1* OR= 1.2 up= 4.4 low= 0.3 n_{min}= 6701
Lung	✗	✗	✗	✗	

Figure 3.2 Co-infection matrix. Ticks indicate a disease pair that occurs together more frequently than expected by chance and crosses indicate those that did not. Associated *p*-values are from Fisher's exact test. Odds ratios (OR) with upper and lower 95%CI (up and low respectively) are also given. The minimum sample size (n_{min}) required is calculated for with 95% probabilities of avoiding type I and type II errors.

From figure 3.2 it is also clear that the statistical power achieved with these tests was inadequate, with no disease combination giving a power of >0.8. As the probability of a type II error is so high, the disease combinations that did not prove significant cannot be considered indicative of an absence of a co-infection relationship. There are some disease combinations that either do not appear in our samples, or occur together at the same frequency alone as in combination. These are the combinations where *p*=1. Skin disease was never observed in an individual that tested positive for CAV-I or *T. gondii*. *T. gondii* and lung disease occurred as frequently together as they did independently. This was also true for the occurrence of lung and kidney disease.

	T.gondii ***positive***	**CAV.I** ***positive***	**Skin** ***positive***	**Kidney** ***positive***	**Lung** ***positive***
T.gondii ***negative***		*p= 0.266* OR= 6.8 up= 112 low= 0.4 n_{min}= 203	*p= 1* OR= 0 up= NaN low= 0 n_{min}= 32	*p= 1* OR= 2.3 up= 58.2 low= 0.9 n_{min}= 31	*p=1* OR= 1 up= 7.9 low= 0.1 n_{min}= 79
CAV.I ***negative***	*p= 0.5234* OR= 7.5 up= 134.6 low= 0.4 n_{min}= 320		*p= 1* OR= 0 up= NaN low= 0 n_{min}= 33	*p= 0.438* OR= 1.9 up= 9.2 low= 0.4 n_{min}= 33	*p= 0.688* OR= 1.7 up= 9.2 low= 0.3 n_{min}= 174
Skin ***negative***	*p= 1* OR= 0 up= NaN low= 0 n_{min}= 28	*p= 1* OR= 0 up= NaN low= 0 n_{min}= 28		*p= 1* OR= 0.6 up= 6.9 low= 0.05 n_{min}= 17	*p= 1* OR= 0.6 up= 8.5 low= 0.04 n_{min}= 54
Kidney ***negative***	*p= 0.524* OR= 4.4 up= 74.7 low= 0.3 n_{min}= 3070	*p= 0.592* OR= 2.8 up= 26.7 low= 0.3 n_{min}= 1235	*p= 1* OR= 0.4 up= 7.9 low= 0.02 n_{min}= 74		*p= 1* OR= 1.1 up= 4.5 low= 0.2 n_{min}>10000
Lung ***negative***	*p= 1* OR= 1 up= 12.4 low= 0.09 n_{min}= 117	*p= 1* OR= 2 up= 17.2 low= 0.2 n_{min}= 281	*p= 1* OR= 0.5 up= 10.6 low= 0.02 n_{min}= 77	*p= 1* OR= 1.2 up= 4.4 low= 0.3 n_{min}= 58	

Figure 3.3 Coinfection matrix of disease parings occurring together less frequently than expected by chance. The *p*-values shown are from Fishers exact test. Odds ratios (OR) with upper and lower 95%CI (up and low respectively) are also given. The minimum sample size (n_{min}) required is calculated for with 95% probabilities of avoiding type I and type II errors.

There is no evidence to suggest that the diseases tested for here competitively exclude one another from hosts, as no disease occurs more frequently than expected in the absence of another (Figure 3.3). The achieved power of these tests is generally higher than when looking for co-infection relationships; but is still frequently inadequate. Where reported power is <0.8 the null hypothesis cannot be accepted even when obtained *p*-values are high.

3.3.3 The relationship between disease and host condition

Figure 3.4 shows the variation in body condition between foxes that were positive and negative for each disease tested. The interaction between gender, age and weight was significant (p=0.002), suggesting that the effect of age on condition depends on weight and gender.

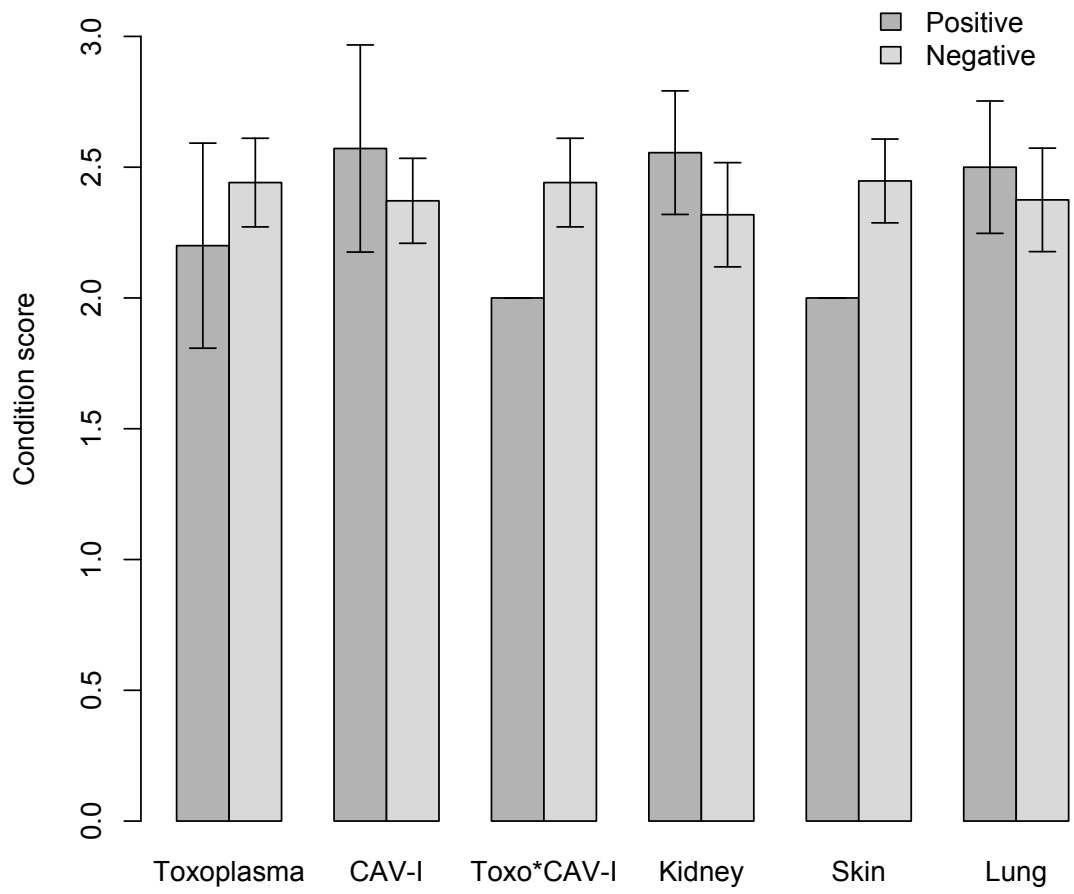

Figure 3.4 Comparing condition scores of foxes that tested positive and negative for each disease. Dark grey bars are positive infection and light grey are negative. Toxo*CAV-I represents individuals with a Toxoplasma and CAV-I co-infection. Error bars show 95% CI.

Foxes exhibiting symptoms consistent with skin infections had lower condition scores than those that did not (p=0.021). The interaction between toxoplasma and CAV-I infection was significant (p=0.0005) showing that foxes suffering from this co-infection were of lower condition than those who were not. Note that all foxes testing positive for both *T. gondii* and CAV-I (n=3) as well as skin pathologies (n=4) had a condition score of 2, hence the lack of confidence intervals around these sample groups (Figure 3.4).

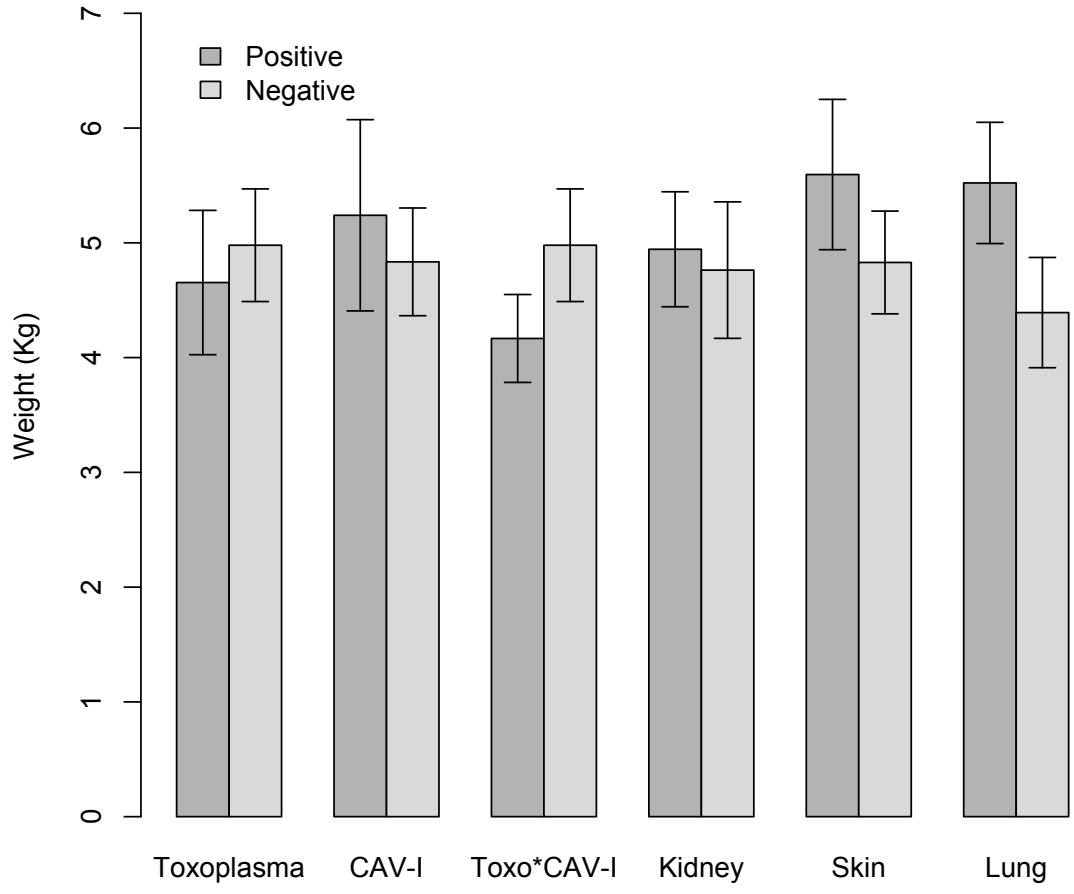

Figure 3.5. Comparing weights of foxes that tested positive and negative for each disease. Dark grey bars are positive infection and light grey are negative. Toxo*CAV-I represents individuals with a Toxoplasma and CAV-I co-infection. Error bars show 95% CI.

Figure 3.5 shows the variation in weight between foxes that were positive and negative for each disease tested. Weight varied significantly with gender and age ($p<0.00004$ and $p<0.000003$ respectively). After controlling for these differences there was no significant relationship between any disease and the weight of individual foxes.

3.4 Discussion

The spatial and behavioural ecology of red foxes in the UK has been remarkably well studied, however the community ecology of the pathogens they play host to has received less attention. Here a multi-disciplinary approach is taken to describe a community of pathogens in a naturally occurring fox population.

Specifically we examined co-infection relationships and the potential impact they have on their environment – the host.

Of the diseases we tested directly for the presence of a pathogen only two were found in our samples. *T. gondii* is present in mammal species across the UK and is well reported. Hamilton et al (2005) reported a toxoplasma prevalence of 20% (n=549) from foxes collected throughout the UK. The prevalence reported here is slightly lower by comparison at 12.82% (n=39). Foxes obtained for Hamilton et al.'s study were from a variety of urban and rural populations. The foxes used in this study were exclusively rural, and thus could be expected to have substantially different dietary habits than their urban counterparts. As *T. gondii* is transmitted via infected prey, and ingestion of under-cooked meat the infection risk of urban and rural populations may differ, which could be partly responsible for the lower prevalence reported here. Thompson et al. (2010) reported the first case of CAV-I infection in free-ranging foxes in the UK, with a prevalence of 19% (n=58) from foxes in Scotland and England. In the population tested here we find a similar prevalence of 16.67% (n=41) using a similar sample size from a more localised area, which supports Thomson et al.'s work and further demonstrates the importance of considering the red fox as a reservoir of this disease which is easily spread to companion animals.

In the population sampled we were unable to find evidence of CDV or CAV-II. Due to the relatively small sample size collected over a period of 17 months it would be unwise to conclude that these diseases absent from the population based on this work alone. However, based on a conservative detection probability it is shown that if either disease is present in the study population it is expected to exist at a prevalence of less than 7%. There is little information in recent scientific literature concerning these two viruses in British populations. Discussing whether we may have expected to find evidence of these infections would be purely speculative. Because of the lack of surveillance for these two pathogens in the UK this negative result is nevertheless important. The survey here adds to a small body of work that suggests that CDV and CAV-II are unlikely to be present at high levels in UK wildlife populations, which is useful in assessing the threat, or lack thereof, these viruses may pose.

Skin, kidney and lung pathologies were examined in this study, as they may be indicative of sarcoptic mange, leptospirosis, or *A. vasorum* infection respectively. The skin pruritus observed on our fox cadavers was certainly consistent with lesions caused by an ecto-parasite, but this does not allow a definitive diagnosis of sarcoptic mange; other skin parasites must be considered as a potential cause. *Demodex spp.* and *Cheyletiella spp.* are mites common in domestic dogs, *Canis familiaris*, in the UK and cause lesions similar to early *S. scabei* infections in appearance. *Dermatophytes* fungal species (ringworm) also cause similar lesions in domestic dogs. Sarcoptic mange infection is well documented in British red foxes, whilst the others aforementioned are not. However, they may not be documented simply because they have not been looked for previously, so we do not rule these other skin infections out and will class these infections simply as skin infections. Evidence of skin disease was observed in 9.52% of the individuals examined and these individuals were of significantly lower condition than individuals not suffering from skin disease. This study cannot discern if individuals of poorer condition are more susceptible to skin infections, or if skin infection causes a poorer condition. However, had it been possible to determine the severity of infection and compare this with host condition a stronger conclusion regarding the cause-effect relationship of this correlation may have been drawn. If these infections were indeed sarcoptic mange, then it is likely that the disease caused a worsening in host condition as this mite is highly contagious and highly pathogenic (Pence & Ueckermann 2002). Whereas Ringworm is primarily an infection of young animals, and elicits a strong and long-lasting immunity upon recovery (Grappel et al. 1974), which would suggest older animals suffering from this infection may have been immune-compromised pre-infection. As we were unable to confirm the pathological agents present on our cadavers as part of this study we are unable to establish the direction of the relationship between skin disease and host condition in this fox population.

The Lung pathology results showed inflammatory aetiologies consistent with a parasite infection (10% prevalence) or an infection by a parasite and/or bacterium (15% prevalence). This would indeed be consistent with our parasite of interest, *A. vasorum*, but further work would need to be carried out to confirm this result. Other potential infections must therefore be considered. The

lung worms *Crenosoma vulpis*, and *Eucoleus aerophilus* have also previously been discovered in the lungs of British foxes (Morgan et al. 2008). These infections would present similar pathologies at post-mortem and so cannot be discounted. Although not much work has been carried out on bacterial respiratory infections of foxes, *Mycobacterium bovis* has been isolated from British foxes previously (Delahay et al. 2001; Delahay et al. 2007). Further testing would be necessary to establish if this was the causative agent of the observed pathologies. It is also possible that lung samples with aetiologies that could not be classed as parasitic or bacterial are indicative of co-infection. Disease of the lung was the only condition studied here where age had an impact on the probability of infection, with yearlings experiencing the highest infection burden. Potential reasons for this may be attributed to: fox denning behaviour, whereby keeping cubs in a den may help limit their exposure to airborne pathogens from hetero- and con-specifics; or waning maternal antibodies leaving foxes of a certain age more susceptible to certain types of infection. *A. vasorum* infections in wild animals are fatal when untreated (Morgan et al. 2008), and so it may be that a combined higher mortality and increased likelihood of infection in young foxes is responsible for this result. It should also be noted that due to the necessary age classing of foxes in this study (pup, yearling, adult) may be masking part of this process – as the "adult" class includes a wider range of fox ages than the other two classes. Foxes may be aged more accurately if a cross section of the canine teeth is taken and rings of enamel counted under a microscope (Harris 1978). This was not done as part of this study, but would certainly be worthwhile in a study with a larger sample size to ensure that statistically significant results were not masked by the method of age classification.

The pathologies observed in the kidneys of the foxes examined were bacterial in aetiology. Whilst this would be in agreement with *Leptospira* infections, other bacterial infections cannot be ruled out. *Borrelia burgdorferi* is known to be present in humans and wildlife in England (Lovett et al. 2008), and although not described in a fox kidney, is known to cause chronic infections in domestic dog kidneys (Grauer et al. 1988) and thus may be considered a candidate pathogen for explaining the pathologies observed. In all likelihood we have observed the results of different bacterial infections in several foxes.

The general pathological symptoms observed here were not found to be involved in co-infection relationships of any kind with any of the pathogens directly tested for. We do however find evidence for a co-infection relationship between *T. gondii* and CAV-I. These two micro-parasites occur together more than expected by chance. Co-infection relationships involving *T. gondii* are known to exist with pathogens that elicit a Th2 immune response (Graham 2002; Jones et al. 2008; Miller et al. 2009) – which are typically macro-parasites. *T. gondii* infection has the potential to cause a long-term Th1 immune response, which is mutually exclusive from the Th2 response needed to combat macro-parasites. This creates a trade-off in immune resources when combatting concomitant infections. Here, the co-infection relationship between two micro-parasites must be utilising a different mechanism. Our results suggest that foxes infected with this combination are of lower quality than foxes carrying one, or neither of these pathogens. Thus one explanation for this co-infection is simply that hosts of lower condition are more prone to infection by multiple micro-parasites. However only this one disease combination remained significant in the GLM employed for this analysis, with all other interaction terms being removed as non-significant. It is equally likely that the poorer condition of these hosts was caused by the co-infection. A further conclusion that may be arrived at is that due to small sample sizes this study is inherently prone to type I error. Indeed, only three individuals tested positive for both CAV-I and *T. gondii*. Although this was enough to detect significance, it would be unwise to extrapolate this result beyond that of the animals tested here based on such a small sample size. The 95% confidence surrounding the odds ratio of this relationship encompasses the value 1: this suggests that if the experiment were repeated we could not be 95% confident of an odds ratio being consistently greater than one. This should be interpreted as an encouraging result that suggests *T. gondii* and CAV-I may naturally co-infect hosts, but further studies should be carried out to provide more confidence in this finding.

The apriori minimum sample size calculations applied to the co-infection data (Figures 3.2 and 3.3) illustrate that future studies should aim for much larger sample sizes in order to be representative of the population from which they were taken. The wide range in sample size calculations and odds ratios is indicative that the strength of co-infection relationships are likely to be highly

variable dependent on the pathogens examined. However, the odds ratios reported here are useful in informing future studies of the expected strengths of these co-infection relationships. The one significant result of this part of the study showing a positive relationship between CAV-I and *T. gondii* infections must be treated cautiously, as the sample size obtained here falls well below that predicted necessary to detect significance, and the 95% confidence intervals surrounding this odds ratio includes one. Therefore this can not be discounted as a chance result.

In this study we have demonstrated methods for use on poorly studied pathogen communities. By utilising both direct and in-direct testing for pathogens we broaden the range of diseases that may be examined as part of a community. Direct testing will allow the identification of specific relationships between pathogens, but relies upon choosing the correct pathogens to test for. Indirect testing has the potential to offer a broader starting point to test for co-occurrence of diseases within a host population, but lacks specificity. By making use of both types of approach we aimed to minimise these limitations. An alternative approach would be to use metagenomics methods. This approach has been highly successful in characterising bacterial diversity from ecosystems (Eisen 2007; Hugenholtz et al. 1998) and has been successfully applied to surveys of human gut bacteria (Backhed et al. 2005). Metagenomics would allow a broad spectrum of disease to be characterised in a population without suffering a lack of specificity, as microbes can be identified molecularly. This method would be less limited by the caveat of only being able to detect what researchers choose to look for.

The pathogen community described here should not be considered definitive. Despite efforts to broaden the scope of the pathogen community examined, ultimately only a small proportion of the pathogens that may be present were considered. Co-infection relationships between gastro-intestinal parasites and mange infections have been shown previously in foxes (Balestrieri et al. 2006). It may be possible that if gastro-intestinal parasites were examined as part of this study more co-infection relationships would have been found. However, considering every pathogen that may be present in a wild host population is a massive, and potentially unfeasible, undertaking. This subset of a disease community examined is supportive of co-infection relationships occurring in

natural populations. More work is needed to establish the impact co-infecting pathogens have on their hosts and the transmission dynamics between hosts. Here it cannot be stated whether co-infecting pathogens are utilising poor quality hosts or are having a more detrimental effect on their host than in a single infection. This study raises questions about the impact co-infection may have on transmission, rather than providing definitive answers. In the example here the host does not transmit *T. gondii*, but it is unknown if this changes the transmission dynamics of CAV-I either by increasing the susceptibility to, or infectiousness of a co-infected host. Despite small sample sizes and only a small subsection of a potential pathogen community being considered, this work gives a useful and interesting insight to the community ecology of pathogens and how pathogen interactions may affect their hosts as well as demonstrating the need for a multi-disciplinary approach to study these communities. Further work exploring pathogen communities will rely on larger sample sizes than presented here, which will involve more intensive cadaver, or sample collection, or a change to a model species which is more abundant and more easily sampled. Future studies utilising modern methods of detection such as metagenomics will undoubtedly prove useful in building upon this work.

4 A model for micro-macro parasite interactions: Could rabies regulate *Echinococcus multilocularis* populations in red foxes?

4.1 Introduction

Disease has long been recognised as a regulatory force of natural populations (Anderson & May 1978; Reddiex et al. 2002; Sait et al. 1994). This usually arises by the pathogen limiting the abundance of the host population. However, in wildlife populations it is the rule rather than the exception that a single population plays host to several pathogens at any point in time. This in effect creates a community of pathogens, which may interact with each other. Examples include: intestinal helminthes competing directly for space (Read 1951), or indirectly through host immune system effects (Cox 2001), or effects caused by differing pathologies and/or transmission strategies employed by different pathogens, for example HIV in humans facilitating infection with other pathogens due to reduced immunocompetence of the host (Bicartsee et al. 1995). Pathogen communities can be thought of broadly on three scales (Bush & Holmes 1986): infra communities consist of different pathogens infecting a single host at any given point in time; component communities are made up of those pathogens found within a population of hosts, and finally compound communities comprise the pathogens infecting a community of host organisms. Currently the importance of community interactions at these three levels is largely unknown, with empirical and theoretical work on the subject still very much in its infancy (but see Bush & Holmes 1986; Fellous & Koella 2010; Graham 2002; Graham et al. 2007; Haukisalmi & Henttonen 1993a; Haukisalmi & Henttonen 1993b; Lanfranchi et al. 2009; Pedersen & Fenton 2007; Read 1951). Looking for empirical evidence of pathogen interactions at the component and compound community levels has thus far relied on correlational studies of co-infection (Balestrieri et al. 2006; Lanfranchi et al. 2009). Whilst important, and undoubtedly useful in proving the existence of such relationships, these studies cannot answer questions regarding the stability and integrity of pathogen communities, or identify if one pathogen is inhibiting or facilitating co-infection. As wildlife disease control becomes more important for conservation and public

health a broader perspective will undoubtedly become imperative to our understanding of the dynamics of disease elimination. Here a simple component community is explored theoretically, using a model based on rabies virus and the cestode Echinococcus multilocularis infecting a wild red fox, Vulpes vulpes, population.

The host, the red fox, is considered the most widespread terrestrial wild carnivore on the planet (McDonald & Reynolds 2004).This small to medium sized canid has proven highly adaptable, and has established large urban populations across Europe, most notably in the UK – where the "urban fox phenomenon" has been observed and reported since the 1930s (Harris & Rayner 1986). Disease is often considered a major demographic pressure on fox populations, with rabies in particular having the potential to decimate natural populations (Pastoret & Brochier 1999). Foxes, like most canines, have a varied diet. Scavenging and predation are however the two primary foraging techniques utilised by this opportunistic omnivore (McDonald & Reynolds 2004). This pre-disposes the fox to many diseases that utilise predator-prey relationships for transmission, including, but not limited to, Toxocara canis, Angiostrongylus vasorum, Toxoplasma gondii, and the parasite of interest *E. multilocularis*. Foxes are territorial and may form loose social groups. Encounters on territory borders together with plastic sociality favours direct transmission of certain diseases such as Canine Distemper Virus (CDV), Canine Adenovirus and Rabies.

Rabies is a zoonotic disease of worldwide importance (Knobel et al. 2005). In the absence of treatment, it is 100% fatal in all carnivora. This disease provides a significant public health and conservation threat worldwide. Across Europe the red fox, Vulpes vulpes, has been implicated as the main reservoir for wildlife rabies since at least 1939, when the current epizootic began in Poland (Holmala & Kauhala 2006). Various culling strategies have been used to control fox populations, but were consistently found to be ineffective. The first trials of orally vaccinating foxes against rabies in Europe began in Switzerland in 1978 (Vitasek 2004), and has been used to great effect ever since. Although rabies is still endemic across much of Eastern Europe several countries across Western and Central Europe have successfully eliminated the disease using oral vaccination (See Vitasek 2004 for a full review). The successful eradication of rabies has had a major impact on the red fox population, causing a large scale

increase in density in both urban and rural settings (Deplazes et al. 2004). This increase in fox population is almost certainly an unwanted side effect, the implications of which still remain unclear.

The small fox tapeworm, *E. multilocularis*, is a parasitic metacestode of canines with a complex life cycle utilising predator-prey relationships between definitive and intermediate hosts for transmission. In Europe the reservoir definitive host for *E. multilocularis* is the red fox, which is infected by the adult stage of the parasite and sheds eggs into the environment via faeces. Arvicolid rodents are the main intermediate hosts, and ingest eggs from the environment. It is at this stage of the life-cycle where incidental hosts, including humans, may be infected. The definitive canine host is infected upon predation of an infected prey animal. There is no mortality associated with this parasite in the definitive host, and infection is regarded as asymptomatic. This parasite is highly non-specific during the larval stage of its life cycle; infecting a variety of mammalian species, including humans, as incidental hosts. The pathology of this parasite in incidental hosts is severe, causing a condition known in humans as alveolar echinococcosis (AE), considered to be the most pathogenic zoonosis in temperate and arctic regions of the Northern hemisphere (Vuitton et al. 2003). Clinical signs of AE can take between five and twenty years to manifest, unless diagnosed and treated with chemotherapy the prognosis is bleak. An undiscovered infection will usually result in fatality (Torgerson et al. 2010).

These two pathogens employ different strategies to maximise their transmission potential, or R_0. Rabies virus achieves this by reproducing quickly within its host. As rabies is transmitted through the bite of an infected animal this high viral load ensures that a sufficiently high viral load is available to be transmitted to the next host, thus ensuring efficient transmission. This strategy of high virulence works to the extreme detriment of the host. In the case of rabies virus the result is death. This is in contrast to the strategy employed by *E. multilocularis*, which requires an intermediate host. When eggs are shed into the environment the parasite has little control over intermediate host predation of individual eggs. So rather than work to a strategy of efficient transmission, *E. multilocularis*, like so many macro-parasites, relies on shedding many eggs over time. The longer it can sustain infection within its definitive host the more eggs it can shed for potential ingestion by intermediate hosts.

From an ecological perspective, these two pathogens are competing with opposing interests, essentially exhibiting different life history strategies and maximizing R_0 in different ways. Infection with rabies, and rapid and inevitable host death will obviously impact negatively on shedding of *E. multilocularis* eggs into the environment. This clearly gives rabies the potential to exert a limiting effect on *E. multilocularis* populations through host removal. Following successful rabies control measures in Europe, there has been an increase both in prevalence of *E. multilocularis* and its geographical range – although the absence of surveys pre-rabies eradication leaves much room for interpretation (Romig 2009). The main driving force behind this would seem to be an increase in available definitive hosts (Deplazes et al. 2004), but due to the lack of surveillance pre- rabies control it is difficult to say with confidence that the increasing fox population alone is wholly responsible for the observed increase in *E. multilocularis* prevalence.

Like so many macroparasites, *E. multilocularis* is highly aggregated in fox populations (Guislan et al. (2008) reported 8% of infected individuals in a fox population in the French Ardennes being responsible for carrying 72% of the total worm biomass). Here we explore the consequences of an interaction between *E. multilocularis* infection and rabies epidemiology as might arise if infection with the macroparasite leads to increased transmission rates of rabies. Specifically, we examine the consequences of a reduction in rabies prevalence as a result of widespread fox-rabies vaccination programs, and their potential impacts on *E. multilocularis* infection loads. This idea is theoretically explored here using a novel model that encompasses the epidemiological dynamics of both a micro and a macro parasite within a dynamic host population. It will be shown that rabies control measures certainly have the potential to lead to an increased population of *E. multilocularis* within the host population. Additionally, we shall examine this limiting effect under the assumptions that it is caused by i) host availability alone or ii) an interaction between worm burden and rabies susceptibility. By varying the transmission coefficient of rabies as a function of worm burden we further investigate the effect of different forms of interaction between the worm burden and rabies susceptibility; examining the effects that rabies may have on aggregation of *E. multilocularis*.

4.2 Methods

We used a deterministic compartmental model that integrated elements from the classic Anderson and May macro-parasite model (Anderson & May 1978; May & Anderson 1978) within a standard Susceptible-Infected-Removed (S-I-R) framework (Anderson & May 1991a). Hosts that were not infected with rabies were assigned to one of *n* discrete classes based on their *E. multilocularis* burden of infection. Rabies susceptible individuals were denoted W_j ($j=0 \ldots n-1$, where W_0 class was uninfected by *E. multilocularis,* and the *j*th class contains P_j worms). Host per capita fecundity, *a*, was assumed to be density dependent, conditional on a host carrying capacity constant, *K*, while per capita background mortality rate, *b*, was assumed to be density independent. Parameters *a, b*, and *K* were assumed to be independent of the *E. multilocularis* burden of infection Intermediate hosts of *E. multilocularis* are not explicitly modelled and transmission is modelled to be environmental - the (in)efficiency of transmission governed by the parameter H_0 (Anderson & May 1978). A fraction of Hosts, f_j, have the potential to enter each class W_j upon infection with *E. multilocularis*, with worms establishing more easily in hosts that "super-spread" the disease, controlled by parameter e_j. The exact mechanism behind the over-dispersion of *E. multilocularis* is unknown; host age, immune function, prey choice and intermediate host susceptibility may all play a part (Guislain et al. 2008; Hofer et al. 2000; Torgerson 2006). It is unknown if, in reality, the worm burden of a fox is determined by burdens in ingested intermediate hosts (so determined at the first infection event) or if subsequent infections allow a build-up of worms in some foxes. In the interest of keeping our model formulation simple, foxes enter a burden category upon infection, in which total worm burden is modelled dynamically. Worms die within their host at rate α, thus also governing the rate of host recovery. parameters were chosen to give a similar aggregation of worms across the *j* categories, as reported by Guislan et al (2008) in the absence of rabies.

The rabies S-I-R model is integrated across this macroparasite model in a straightforward way except that we allow for the possibility that the rabies transmission coefficient β_j is dependent on the burden of *E. multilocularis* infection. Rabid hosts die at rate at $b+\gamma$. The dynamics are thus governed by the

following equations, where W_{Total} equals the sum of all W; W_{EM} are only those foxes infected with *E. multilocularis* and is thus the sum of all W excluding W_0; and P is the total parasite biomass and is the sum of all $W_j i_j$:

$$\frac{dW_0}{dt} = a\left(W_{Total} + I\right).\left(\frac{K - (W_{Total} + I)}{K}\right) - bW_0 \ldots$$
$$\ldots - \beta_0 W_0 I - \left(\frac{\lambda P W_{EM}}{H_0 + W_{EM}}\right).\left(\frac{W_0}{W_{total}}\right) \quad \textbf{Equation 4.1.1}$$

$$\frac{dW_1}{dt} = \left(\frac{\lambda P W_{EM}}{H_0 + W_{EM}}\right).\left(\frac{W_0}{W_{total}}\right) - bW_1 - \sigma_1 W_1 - \beta_1 W_1 I \quad \textbf{Equation 4.1.2}$$

$$\frac{dW_2}{dt} = \sigma_1 W_1 - bW_2 - \sigma_2 W_2 - \beta_2 W_2 I \quad \textbf{Equation 4.1.3}$$

$$\frac{dW_n}{dt} = \sigma_{(n-1)} W_{(n-1)} - bW_n - \sigma_n W_n - \beta_n W_n I \quad \textbf{Equation 4.1.4}$$

$$\frac{dI}{dt} = \sum_{j=0}^{n} \beta_j W_j I - bI - \gamma I \quad \textbf{Equation 4.1.5}$$

$$\frac{dR}{dt} = \gamma I \quad \textbf{Equation 4.1.6}$$

To model an interaction between worm burden and rabies susceptibility β_j was modelled as a function of worm burden, i, whilst constrained to give an R_0 of 1.4. Given that rabies' R_0 for the system as a whole is explained by equation 4.2, transmission heterogeneity can be achieved with the following equations:

$$R_0 = \sum_j \frac{\beta_j W_j}{b + \gamma} \quad \textbf{Equation 4.2}$$

$$\beta_j = x\left(1 + \frac{f(i_j)}{z}\right) \quad \textbf{Equation 4.3}$$

The constant z is inversely proportional to the enhanced rabies transmission caused by each worm in maximally burdened foxes. It is manipulated as:

$$z = \frac{i_{\max}}{strength} \quad \textbf{Equation 4.4}$$

Varying "strength" allows a tangible manipulation of β_j in response to heterogeneity in *E. multilocularis* burdens. The term "strength" represents how many more times greater β_j is in the highest worm category compared to in W_0. Substituting equation 4.3 into equation 4.2 leads to an expression that can be solved for x, which can then be used to calculate β_j for each class, W_j, of rabies-susceptible foxes. When equation 4.4 is substituted into equation 4.3, the term "strength" informs how many times higher the transmission of rabies to foxes with the maximum worm burden is compared to foxes without worms.

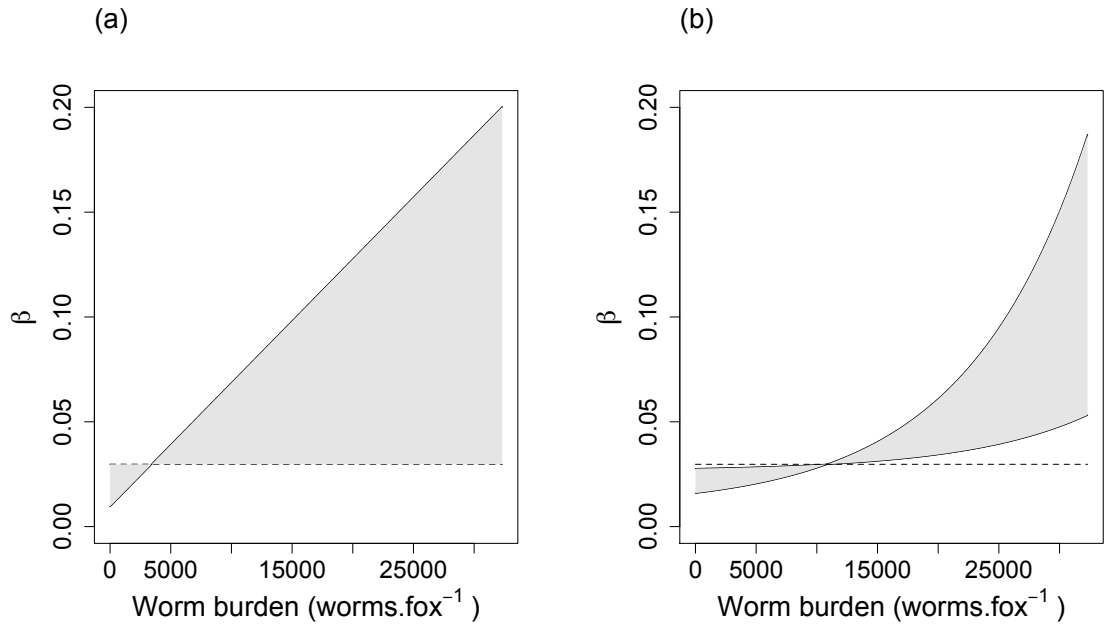

Figure 4.1 Variation of the rabies transmission co-efficient β as (a) linear and (b) exponential functions of worm burden. Broken line illustrates the null hypothesis (interaction strength =1) and shaded areas represent the areas of parameter space where manipulations were performed (interaction strength = 1.5 to 20).

Two simple functions of worm burden were tested, representing two different versions of our alternate hypothesis: β_j increasing linearly as a function of worm burden, *i*:

$$f(i) = i_j \qquad \textbf{Equation 4.5}$$

and β_j increasing exponentially as a function of worm burden:

$$f(i) = \exp[ci_j] \qquad \textbf{Equation 4.6}$$

The former assumes that each worm has an equal effect on its host, whilst the latter assumes that heavily infected foxes are disproportionally more likely to acquire rabies compared to those with a low to average worm burdens, which suffer a much lesser additional susceptibility. The parameter c in equation 4.6 is a scaling constant held fixed to $1x10^{-4}$ across all manipulations which was necessary to avoid computational errors which arose from dealing with the exponents of extremely large numbers associated with heavy worm burdens.

As well as examining different forms of potential relationship between rabies and worm burden, differing modes of rabies transmission were also examined: the model outlined in equations 4.1.1-4.1.5 uses a density dependent transmission term to simulate rabies dynamics. To model this system with frequency dependent dynamics the rabies transmission term "$\beta W_j I$" was substituted for "$(\beta_j W_j I) / (W_j + I)$". The R_0 formulation in equation 4.7 was used in calculating the transmission co-efficient for these simulations:

$$R_0 = \sum_j \frac{\beta_j}{b+\gamma} \qquad \textbf{Equation 4.7}$$

The model was explored numerically by simulating from initial conditions to equilibrium for the scenarios demonstrated in figure 4.1. Figure 4.1(a) demonstrates the response of increasing interaction strength when β_j is linearly dependent on worm burden (equation 4.5). As interaction strength increases the difference between the intercept and maximum value of β_j becomes greater, so more heterogeneity in rabies transmission is introduced. Figure 4.1(b) demonstrates a similar pattern, showing that increasing interaction strength increases heterogeneity in transmission. This scenario differs from the previous in that varying interaction strength has a smaller effect on foxes with low worm burdens. The intercept for the exponential function is higher than when a linear interaction is used with the same interaction strength. All other model parameters are listed in table 1 with their assigned values.

symbol	parameter	unit	value	basis
a	fox birth rate	foxes day^{-1}	4.53x10^{-3}	(Harris & Smith 1987)
b	fox mortality rate	foxes day^{-1}	2x10^{-3}	(Takumi & Van der Giessen 2005)
K	carrying capacity constant	constant	12	Estimated so equilibrium density in the absence of rabies is 6.6903km^{-2}
β	rabies transmission coefficient	new infections infective^{-1} susceptible $^{-1}$day^{-1}	variable	Based on R_0 of 1.4
γ	rabies associated mortality rate	foxes day^{-1}	0.14	Based on a 7 day infectious period
λ	worm birth rate	eggs day^{-1}worm^{-1}	42.00	(Takumi & Van der Giessen 2005)
H_0	transmission inefficiency	fox	3259.89	Calculated as in (Anderson & May 1978)
i_j	worm burden in class W_j	worms fox^{-1}	[0; 20; 427; 3221; 32309]	Calculated from (Guislain et al. 2008)
σ_1	rate of progression from $W_1 \rightarrow W_2$	foxes day^{-1}	2.79 x 10^{-3}	At equilibrium RF state 24.0% of infected foxes are in class W_2 (Guislain et al. 2008)
σ_2	rate of progression from $W_2 \rightarrow W_3$	foxes day^{-1}	2.84 x 10^{-3}	At equilibrium RF state 26.6% of infected foxes are in class W_3 (Guislain et al. 2008)
σ_3	rate of progression from $W_3 \rightarrow W_4$	foxes day^{-1}	5.71 x 10^{-4}	At equilibrium RF state 7.6% of infected foxes are in class W_4 (Guislain et al. 2008)

Table 4.1 Definitions of parameters and assigned values. RF refers to 'rabies free' state.

4.3 Results

4.3.1 Does rabies virus regulate *E. multilocularis* populations through limiting host availability?

In the absence of rabies both fox and worm populations reach their maximum values at equilibrium. In Table 4.2 these equilibrium values are compared to the equilibrium values in the presence of rabies, in the absence of an interaction between rabies and *E. multilocularis* infection. Equilibrium values of both worm and fox populations are appreciably lower in the presence of rabies.

	rabies free state	rabies present under null hypothesis	difference (%)
fox density (km^{-2})	6.69	5.004	28.58
worm biomass (km^{-2})	22888.09	10748.05	53.04
mean worm burden	3421.09	2249.30	34.25

Table 4.2 Equilibrium populations of foxes and *E. multilocularis* under rabies free and null hypothesis conditions.

In rabies endemic areas the fox density is predicted to be 28.6% lower than if rabies is absent. However the reduction in equilibrium worm biomass (worms km^{-2}) in the presence of rabies is lower by 53.0%. The reason for this is when rabies is present in our model fewer foxes survive long enough to acquire higher worm burdens. This is illustrated by the fact that average worm burden of individual foxes is about 34.3% lower in the presence of rabies. It can therefore be seen that both the total environmental *E. multilocularis* burden and its aggregation amongst hosts are limited by the demographic pressures rabies exerts on red fox populations.

4.3.2 Does a linear interaction between worm burden and rabies susceptibility further limit *E. multilocularis* populations? And could it effect the over-dispersion of worms amongst hosts?

By representing the rabies transmission coefficient in our model as a function of worm burden we explore an interaction between the two pathogens. This

interaction is modelled at varying strengths representing an increasing effect of worm burden on rabies transmission. The stronger this interaction the more heterogeneity in rabies transmission experienced by the population, with a larger deviance either side of the mean value of β_j. The linear distribution assumes a relationship between worm burden and rabies susceptibility where each individual worm has an equally increases the probability of rabies infection of its host.

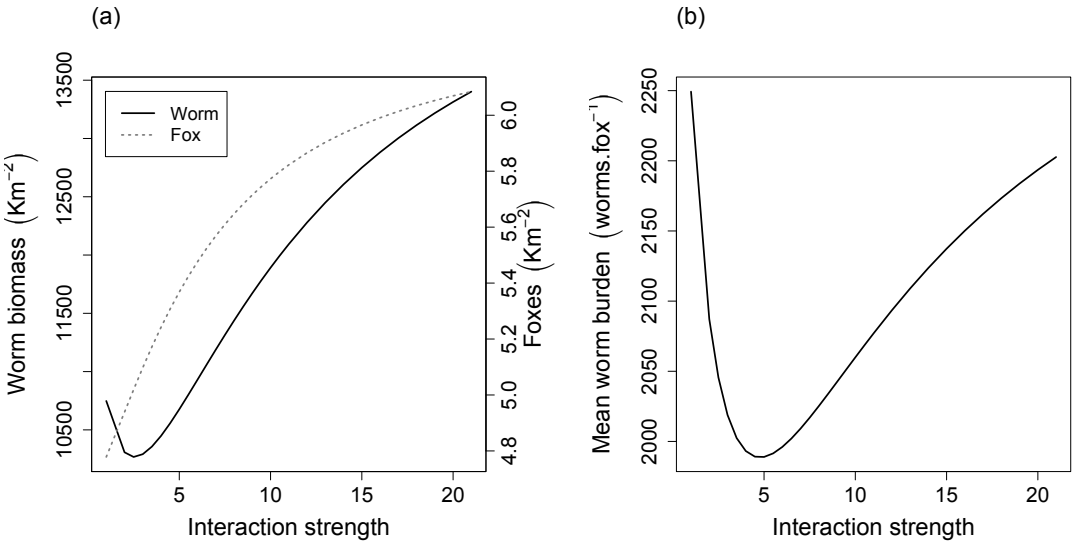

Figure 4.2 Equilibrium population densities at varying interaction strengths when β_j is modelled as a linear function of worm burden under density dependent transmission. (a) changes in both fox density and worm biomass; (b) Changes in mean worm burden

It can be seen from Figure 4.2(a) that weaker interactions have the most severe effect on worm biomass. Suppression is strongest at an interaction strength of 2.5, at which point the *per capita* rabies transmission to foxes with the maximum worm burden is 2.5 times higher than to foxes without worms. This results in an equilibrium *E. multilocularis* biomass that is 4.5% less than in the absence of an interaction, and 55.1% less than in the absence of rabies. Further increases in interaction strength caused equilibrium worm biomass to increase. At an interaction strength of 5.5, at which point *per capita* rabies transmission to foxes with the maximum worm burden is 5.5 times higher than to foxes without worms, equilibrium worm biomass was higher than that under the conditions of the null hypothesis. The increased heterogeneity in rabies transmission at higher interaction strengths benefits *E. multilocularis* populations, resulting in higher equilibrium biomass of worms. Figure 4.2(b)

shows the minimum mean worm burden experienced by foxes occurs at a higher interaction strength than the minimum total biomass. This was at an interaction strength of 5 with a mean worm burden 11.6% lower than in the absence of an interaction. In contrast to total worm biomass, when β_j is modelled as a linear function of worm burden, equilibrium mean worm burden never becomes higher than in the absence of an interaction. This difference is indicative of rabies affecting both *E. multilocularis* abundance through host limitation and aggregation by preferentially infecting foxes with higher worm burdens.

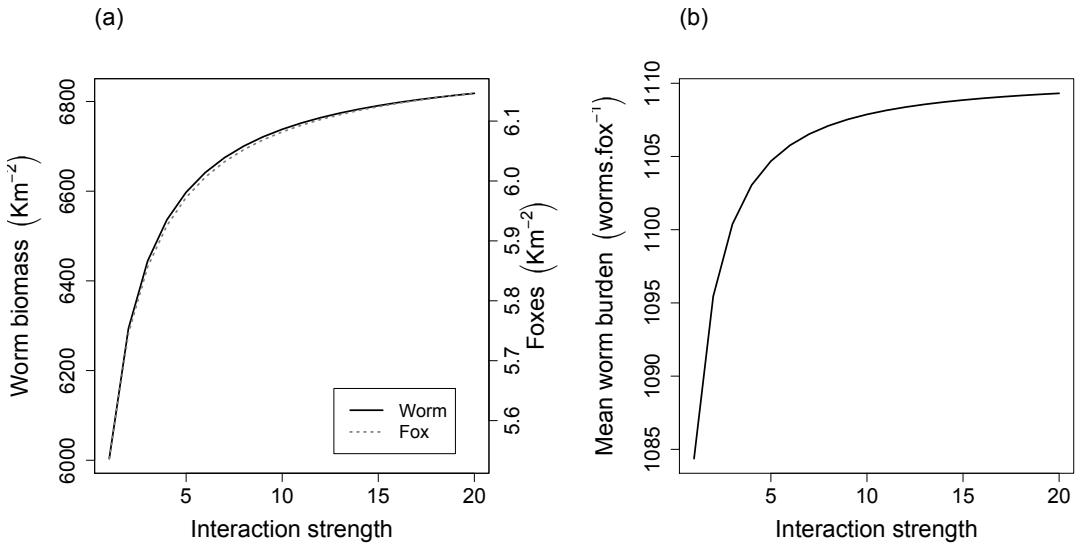

Figure 4.3 Equilibrium population densities at varying interaction strengths when β_j is modelled as a linear function of worm burden under frequency dependent transmission. (a) Changes in both fox density and worm biomass; (b) Changes in mean worm burden.

When rabies is modelled with frequency dependent transmission and a linear dependency on worm burden, increases in interaction strength cause rabies to become less suppressive on both worm and fox populations, as demonstrated by Figure 4.3. However, when comparing Figures 4.2 and 4.3 it becomes clear that when modelled with frequency dependent transmission, rabies is more suppressive on worm populations than when modelled with density dependent transmission, and marginally less suppressive of fox populations. At the maximum interaction strength of 20 when rabies is modelled with frequency dependent transmission total worm biomass Km^{-2} is 47% lower than when the same strength is used to model rabies with density dependent transmission, while fox density is marginally higher by 0.4%. Qualitatively, the two

transmission modes yield similar results for fox density, but worm biomass is more sensitive to changes in transmission mode under this linear relationship between rabies transmission and *E. multilocularis* burden.

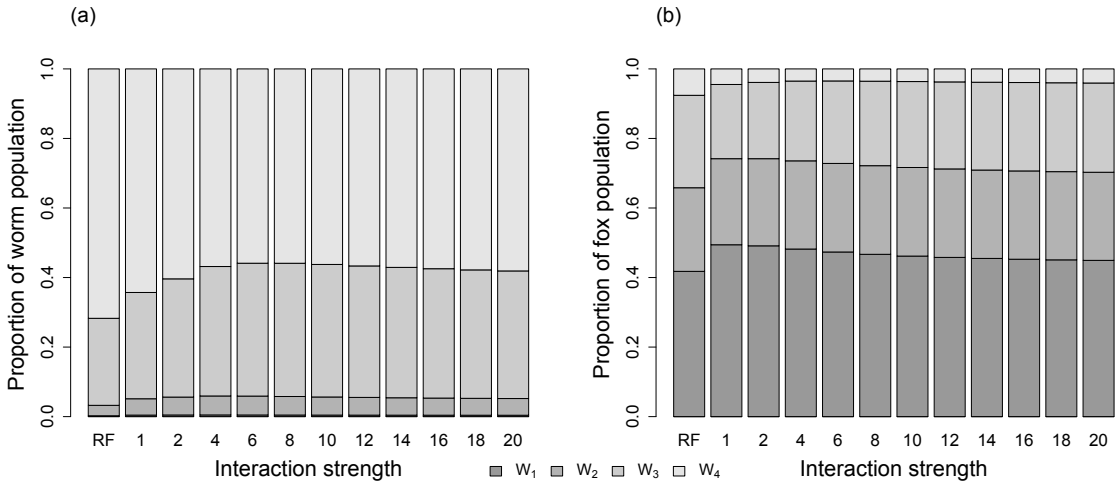

Figure 4.4 Equilibrium aggregation of worms at different interaction strengths when β_j is modelled as a linear function of worm burden under density dependent rabies transmission. (a) The proportion of worms in each infected W class; (b) The proportion of foxes in each infected W class. "RF" denotes the rabies free equilibrium values and an interaction strength of 1 is equal to the null hypothesis conditions in the presence of rabies.

It can be seen in Figure 4.4(a) the proportion of worms in each class ($W_1 \rightarrow W_4$) changes in response to changes in interaction strength. In the absence of rabies 72% of worm biomass is in W_4. Introduction of rabies causes this to fall to 64%, and a minimum of 56% at an interaction strength of 7. The proportion of worms in W_4 increases thereafter, but does not reach the levels obtained in the absence of an interaction. In the absence of rabies 7.5% of foxes are in W_4. Introduction of rabies causes this to fall to 4.5%, and a minimum of 3.5% is reached at an interaction strength of 5.5. The proportion of foxes in W_4 increases thereafter, but does not reach the levels obtained in the absence of an interaction, as illustrated by Figure 4.4(b).

When Figure 4.4 is compared with Figure 4.5 it can be seen that when rabies is modelled with frequency dependent transmission that the proportion of worms in class W_4 is greatly diminished, as too is the proportion of foxes. At an interaction strength of two only 22% of worms are contained in W_4 (compared to 72% in the rabies free state), and by an interaction strength of 20 only 9% of worms are contained in this class. At an interaction strength of 0 (homogenous

transmission across all classes), only 21% of worms are in contained in W_4. This suggests that if rabies is frequency dependently transmitted then it has the potential to switch *E. multilocularis* from an over-dispersed population structure to being under-dispersed.

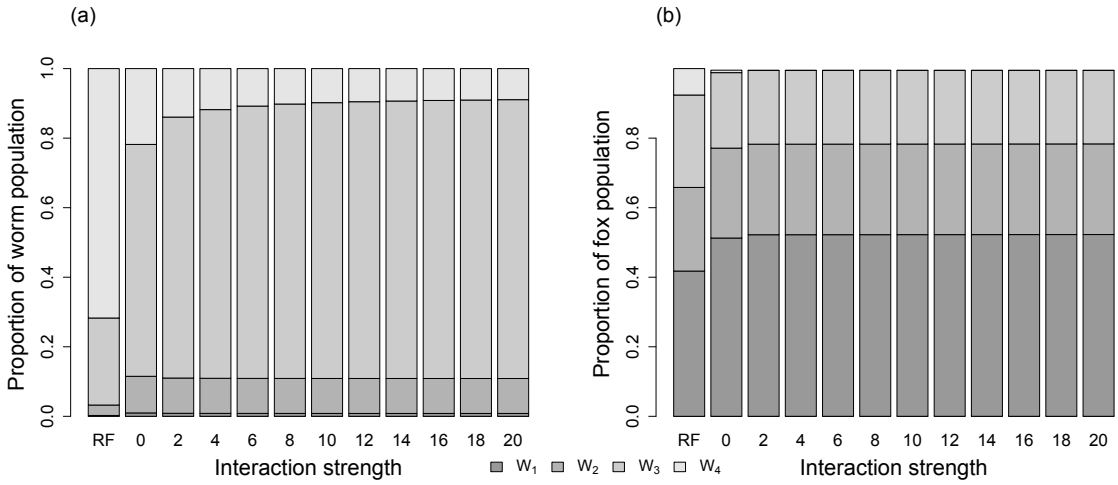

Figure 4.5 Equilibrium aggregation of worms at different interaction strengths when beta is modelled as a linear function of worm burden with frequency dependent rabies transmission. (a) The proportion of worms in each infected W class; (b) The proportion of foxes in each infected W class. "RF" denotes the rabies free equilibrium values.

Low strength linear interactions between worm burden and rabies susceptibility have the potential to regulate both the total population of *E. multilocularis* as well as the over-dispersion of worms amongst hosts than under the conditions of the null hypothesis. When rabies is modelled with density dependent transmission, at medium to high interaction strengths, a linear interaction between rabies susceptibility and worm burden also has the potential to reduce the regulatory impact of rabies on *E. multilocularis*. Whereas if rabies is modelled with frequency dependent transmission *E. multilocularis* is heavily supressed and although increases in interaction strength cause a slight increase in total *E. multilocularis* biomass, the over-dispersion of worms remains greatly supressed.

4.3.3 Do different forms of interaction between worm burden and rabies susceptibility differently affect *E. multilocularis* population size and over-dispersion amongst hosts?

We considered that the form of interaction between worm burden and rabies susceptibility might have further effects on the regulation of *E. multilocularis*. Replacing β_j in our model with an exponential function results in those foxes with low to average worm burdens being mildly less susceptible to rabies than average, and the minority of foxes with extremely high worm burdens much more susceptible.

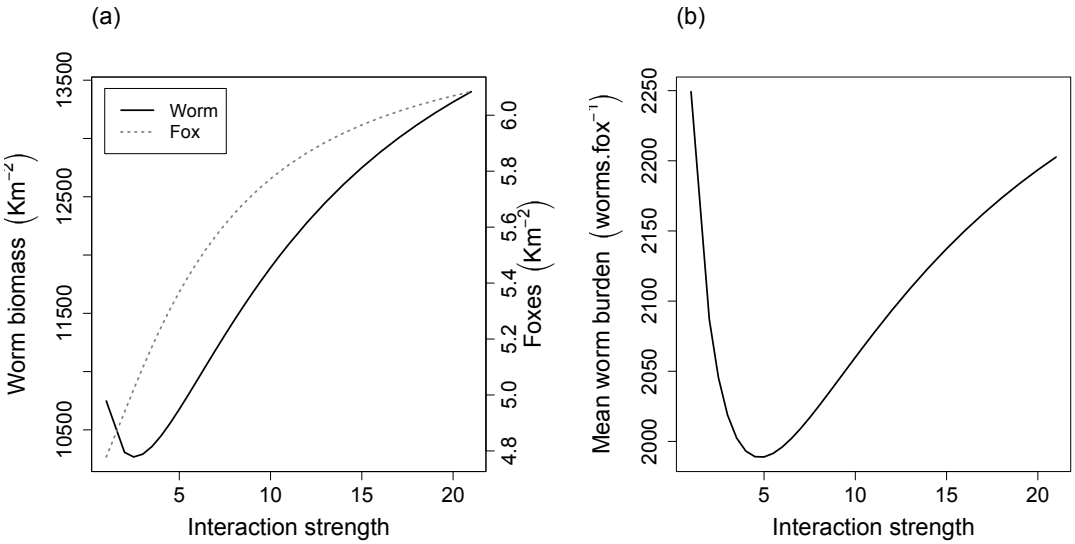

Figure 4.6 Equilibrium population densities at varying interaction strengths when β_j is modelled as an exponential function of worm burden. (a) Changes in both fox density and worm biomass; (b) Changes in mean worm burden.

Both Fox and *E. multilocularis* populations behave similarly as when exposed to a linear function of worm burden on β_j. This is apparent when comparing figures 4.3 and 4.6, which are remarkably alike. It can be seen in Figure 4.6(a) that the minimum biomass of worms occurs at an interaction strength of 2.5, with a reduction of 4.5% in biomass compared to that predicted under the conditions of the null hypothesis and 55.1% than in the absence of rabies. At an interaction strength of 5.5 the equilibrium biomass of worms becomes higher than in the absence of an interaction. In Figure 4.6(b) it can be seen that the minimum mean worm burden occurs at an interaction strength of 5 and is 11.6% lower than in the absence of an interaction. As was seen when β_j was modelled as a linear function of worm burden, equilibrium mean worm burden continues to rise

after this minimum, but does not surpass the levels observed in the absence of an interaction.

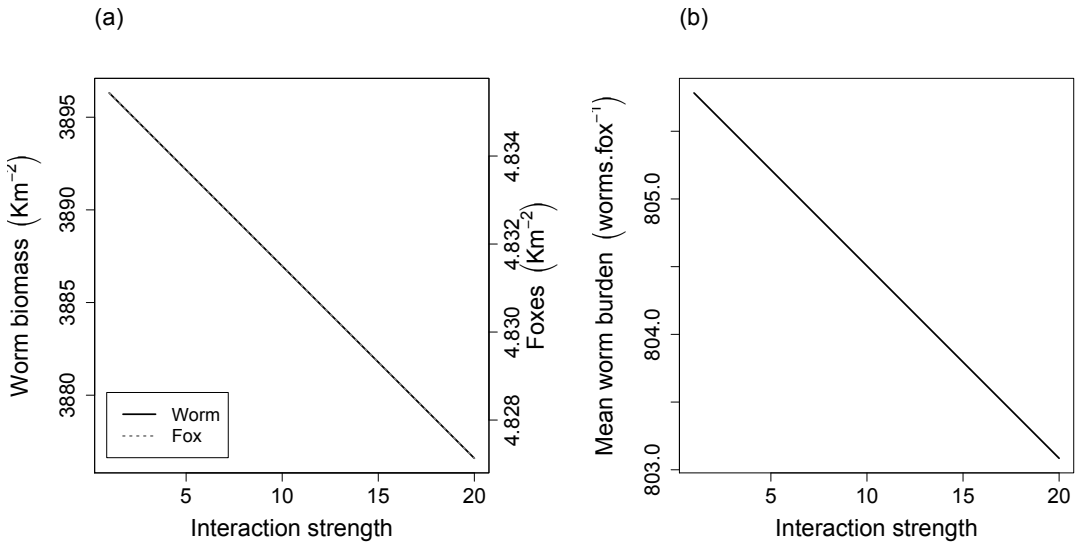

Figure 4.7 Equilibrium population densities at varying interaction strengths when β_j is modelled as an exponential function of worm burden with frequency dependent rabies transmission. (a) Changes in both fox density and worm biomass. (b) Changes in mean worm burden.

When rabies is modelled with frequency dependent transmission and β_j is an exponential function of worm burden increasing interaction strength causes rabies to be marginally more suppressive on worm and fox populations, as shown by Figure 4.7, noting the small ranges on all y axes. Both Worm and Fox populations show a linear decrease in size at equilibrium as interaction strength is increased.

Figure 4.8 demonstrates that the aggregation of worms amongst foxes when β_j is modelled exponentially is remarkably similar as to when modelled linearly. Figure 4.8(a) illustrates how the proportion of worms is each class ($W_1 \rightarrow W_4$). Equilibrium aggregation in the absence of rabies and in the presence of rabies with no transmission heterogeneity is the same as Figure 4.4. The minimum proportion of worms in class W_4 occurs at an interaction strength of 8, where 55.2% of worms are found in this class. This is only slightly lower than the minimum of 56% obtained by modelling β_j linearly, and occurs at a higher interaction strength.

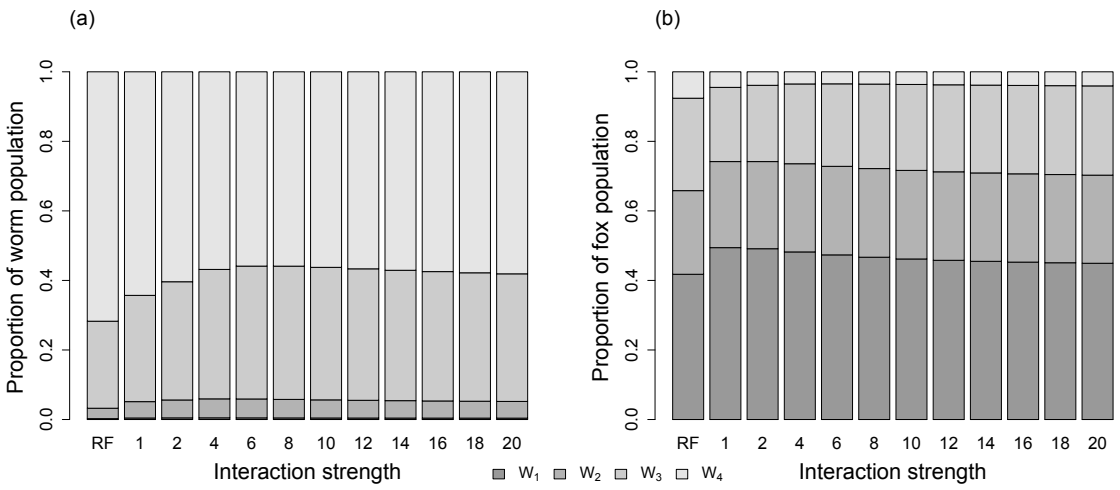

Figure 4.8 Equilibrium aggregation of worms at different interaction strengths when β_j is modelled as an exponential function of worm burden. (a) The proportion of worms in each infected W class; (b) The proportion of foxes in each infected W class. "RF" denotes the rabies free equilibrium values.

The equilibrium proportion of worms in W_4 remains marginally lower for the exponential function than the linear for all interaction strengths above this minimum. Equilibrium fox densities in each class ($W_1 \rightarrow W_4$) are shown in Figure 4.8(b). The minimum density of foxes in W_4 is 3.4% occurs at an interaction strength of 6. This is again marginally lower than the minimum obtained when β_j was modelled as a linear function, and occurs at a higher interaction strength. Equilibrium density of foxes in W_4 remains marginally lower than when a linear function of β_j is modelled for interaction strengths above this minimum.

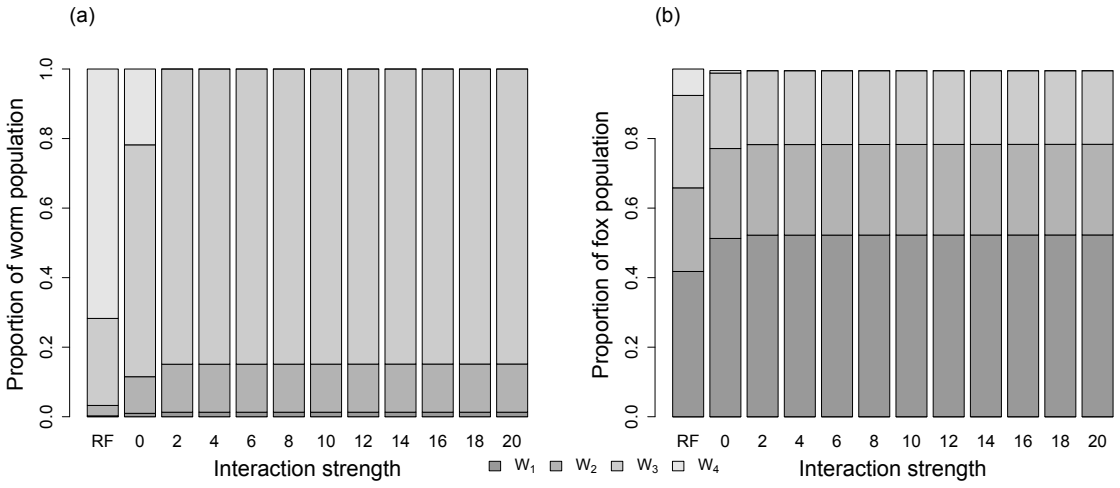

Figure 4.9 Equilibrium aggregation of worms at different interaction strengths when β_j is modelled as an exponential function of worm burden with frequency dependent rabies transmission. (a) The proportion of worms in each infected W class; (b) The proportion of foxes in each infected W class. "RF" denotes the rabies free equilibrium values.

When β_j is modelled with as an exponential function of worm burden under frequency dependent rabies transmission dynamics *E. multilocularis* aggregation is very strongly affected. It is shown in Figure 4.9(a) that introducing this form of heterogeneity in rabies transmission when modelled with frequency dependent dynamics instantly causes the proportion of worms contained in class W_4 to approach 0, while 85% of the worm population are contained in class W_3. This result changes very little (<0.001%) across the interaction strengths ranging from one to twenty. Whilst the *E. multilocularis* population can still be considered over-dispersed, as class W_4 is functionally extinct, and the bulk of worms now existing in class W_4, the majority of the most heavily infected foxes now have a reduction in worm burden of about ten orders of magnitude.

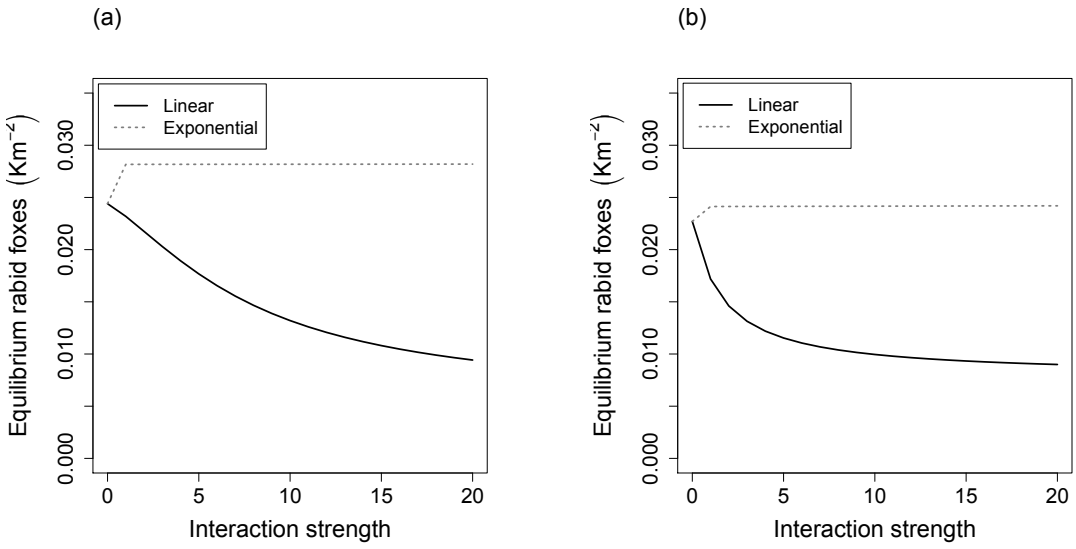

Figure 4.10 Equilibrium densities of rabid foxes across a range of interaction strengths for both linear and exponential functions of rabies transmission. (a) Density dependent rabies transmission; (b) Frequency dependent rabies transmission.

Figure 4.10 shows the difference in densities of rabid individuals between the exponential and linear functions at equilibrium increases as interaction strength increases. Increasing interaction strength when a linear function of worm burden is used to describe rabies transmission leads to a lower density of rabid foxes for both the density dependent and frequency dependent transmission scenarios. Whereas when an exponential function of worm burden is used to describe rabies transmission introducing there is an increase in the equilibrium density of rabid foxes that remains constant across the interaction strengths tested. At an

interaction strength of zero (the null hypothesis conditions) all scenarios yield the same equilibrium density of rabid foxes.

4.3.4 Relaxing the assumption of a constant R_0

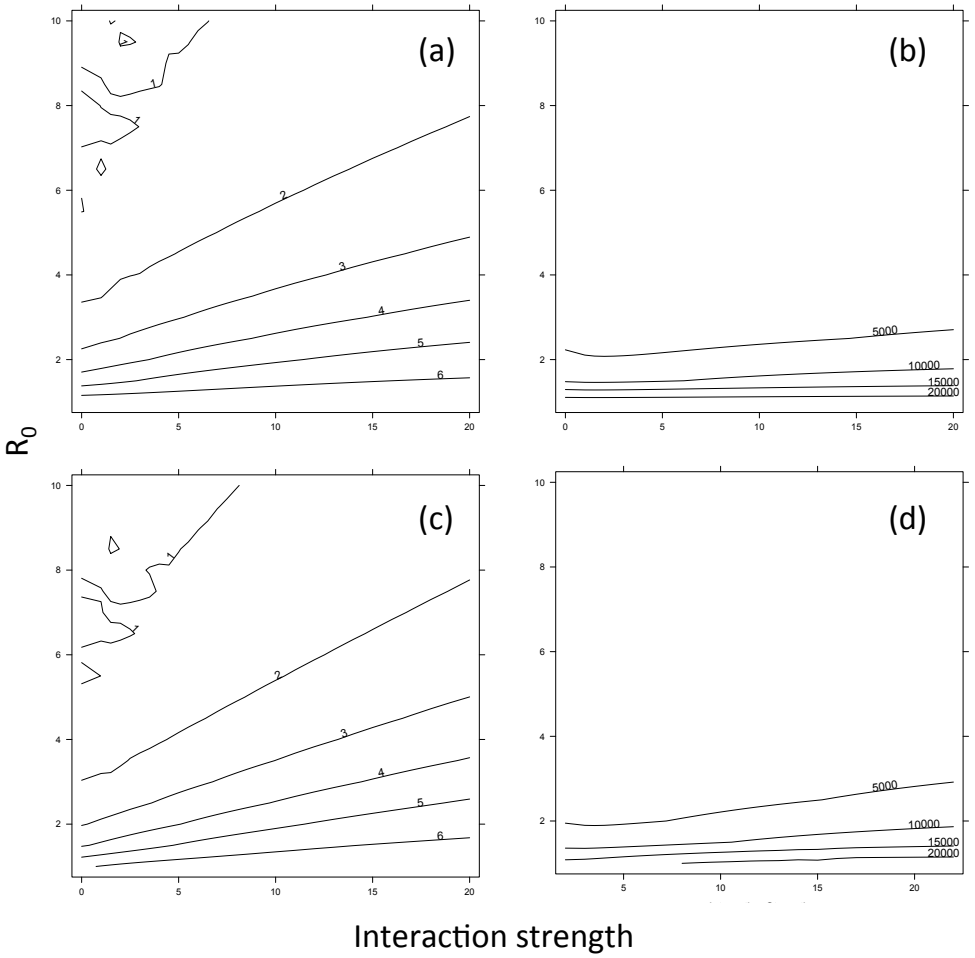

Figure 4.11 Contour plots showing the sensitivity of fox density and *E. multilocularis* biomass to changes in interaction strength and Rabies R_0 when rabies is modelled with frequency dependent transmission. Contour lines show: (a) Fox density (km^{-2}) when β_j is modelled as a linear function of worm burden; (b) *E. multilocularis* biomass (worm^{-1}km^{-2}) when β_j is modelled as a linear function of worm burden; (c) Fox density (km^{-2}) when β_j is modelled as an exponential function of worm burden; (d) *E. multilocularis* biomass (worm^{-1}km^{-2}) when β_j is modelled as an exponential function of worm burden.

Figure 4.11 illustrates the effects of relaxing the assumption of increasing interaction strengths across a variety of R_0 values when rabies is modelled with density dependent transmission. The linear (Figure 4.7(a.b)) and exponential (Figure 4.7(c,d)) manipulations of β again show very similar effects on fox density (Figure 4.7(a,c)) and *E. multilocularis* biomass. As R_0 is increased, the

effect of increasing interaction strength becomes more beneficial to fox density, but overall systems where R_0 of rabies is higher fox density is lower.*E. multilocularis* biomass (Figure 4.7(b,d)) is fairly insensitive to interaction strengths, with low interaction strengths only having a slightly more suppressive effect across all values of R_0. In comparison *E. multilocularis* is very sensitive to changes in the R_0 of rabies. Absolute values of a competing pathogens R_0 are thus more important for suppressing *E .multilocularis* populations.

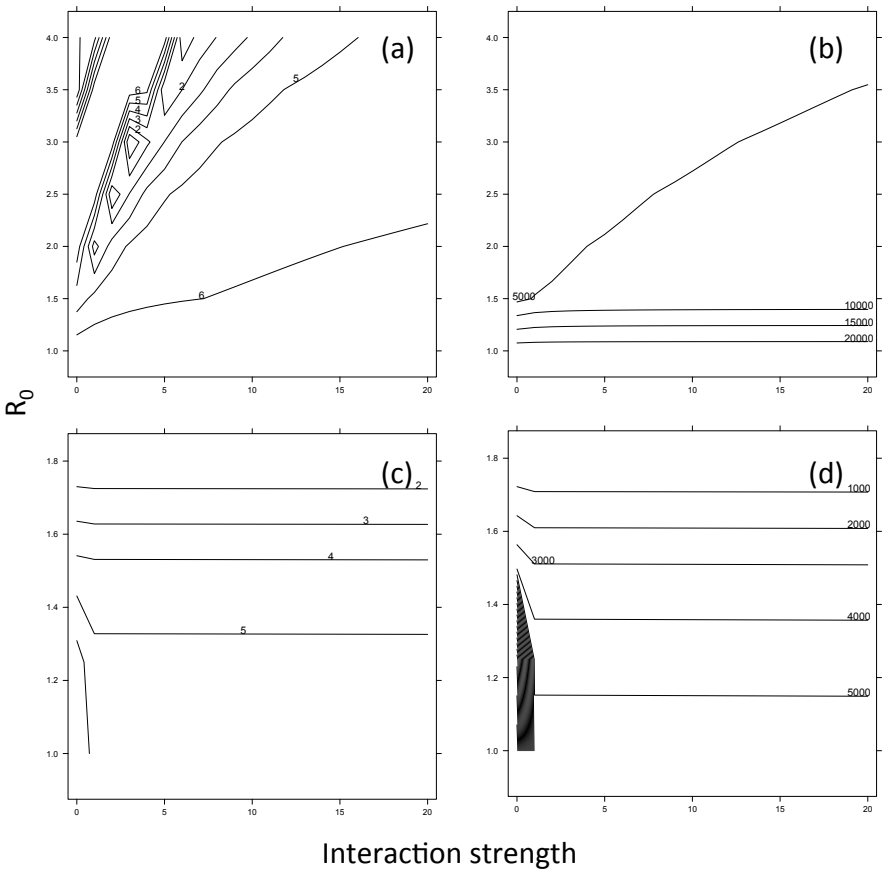

Figure 4.12 Contour plots showing the sensitivity of fox density and *E. multilocularis* biomass to changes in interaction strength and rabies R_0 when rabies is modelled with frequency dependent transmission. Contour lines show: (a) Fox density (km^{-2}) when β_j is modelled as a linear function of worm burden; (b) *E. multilocularis* biomass ($worm^{-1}km^{-2}$) when β_j is modelled as a linear function of worm burden; (c) Fox density (km^{-2}) when β_j is modelled as an exponential function of worm burden; (d) *E. multilocularis* biomass ($worm^{-1}km^{-2}$) when β_j is modelled as an exponential function of worm burden.

Figure 4.12 illustrates the effects of relaxing the assumption of increasing interaction strengths across a variety of R_0 values when rabies is modelled with frequency dependent transmission. Figures 4.12 (a,c) show that the reaction of equilibrium fox densities to these manipulations differ depending on whether a linear or exponential function of worm burden is used to describe rabies

transmission. Increasing R_0 may lead to an increase or decrease in fox population density dependent on interaction strength. Whereas when the exponential function is used, increasing R_0 always leads to a decrease in fox density, which is relatively robust to changes in interaction strength. Figures 4.12(b,d) demonstrate that when rabies is modelled with frequency dependent transmission worm biomass always decreases with an increasing rabies R_0.

4.4 Discussion

This work gives a new perspective to the current emergence of *E. multilocularis* occurring in Europe by demonstrating that rabies virus has the potential to regulate total *E. multilocularis* biomass and aggregation through host availability. Rabies virus may have a further, small, regulatory effect on *E. multilocularis* biomass and aggregation if the worm burden of hosts causes low levels of heterogeneity in rabies transmission, and also dependent on the transmission dynamics rabies exhibits: differing between frequency and density dependent dynamics. Mechanisms behind an interaction behind rabies virus and *E. multilocularis* are difficult to demonstrate empirically due to a lack of surveillance data, so whilst mechanisms may be tested theoretically, the exact causes of such a relationship are speculative. One explanation could be a direct effect on the immune system of the host: A high infection load of macro parasites would cause a maintained Th2 humeral response, making the host less able to elicit the Th1 response needed to combat a rabies infection (Cox 2001; Graham et al. 2007). However, as there is no convincing evidence to suggest foxes ever having immunity to rabies, this mechanism seems unlikely. A second potential mechanism could stem from the physiological state of individual foxes. As previously stated, *E. multilocularis* is considered asymptomatic. However, it does not seem unreasonable to suppose that those foxes carrying the majority of the parasite population would at the very least begin to suffer effects of malnutrition and anaemia generally associated with gastro-intestinal parasite infections. In order to counter this effect, heavily infected foxes could be assumed to be increasing their foraging effort, which would likely involve travelling a further distance to locate additional prey, and an increased probability of making extra-territorial excursions. If this was the case, it would certainly increase the individuals contact rate with other foxes, and this would in turn increase the chance that this heavily infected individual will encounter a

rabid conspecific. Given what is already known about fox ecology, this seems more likely than a direct immunological effect.

Under the null hypothesis there is no interaction between worm burden and rabies susceptibility. These conditions assume the only limitation placed on *E. multilocularis* populations is the availability of definitive hosts. The proportion of foxes infected with *E. multilocularis* remains unchanged between the null hypothesis and rabies free state when rabies is modelled with density dependent transmission. This is because the force of infection for *E. multilocularis* is linearly related to the density of hosts; so while the density of hosts infected with *E. multilocularis* changes, the proportion of the host population infected is constant. This limits the total biomass of *E. multilocularis* through host availability. However if rabies is modelled with frequency dependent transmission this relationship is lost, and rabies does limit the over-dispersion of *E. multilocularis*, even in the absence of an interaction. Recent work tracking rabies transmission in domestic dogs (Hampson et al. 2009) suggests rabies is transmitted with frequency dependent dynamics. Whilst it should not be assumed to follow the same dynamics in wild populations of foxes, it is sensible to postulate that transmission dynamics may be closer to frequency dependence than density dependence. In light of this it seems likely that rabies has the potential to supress both prevalence, and aggregation of *E. multilocularis* in its natural host populations of foxes.

The lower fox density found in the presence of rabies hosts a lower total biomass of *E. multilocularis*. This clearly illustrates that definitive host availability is an important limitation on *E. multilocularis* populations. Further to this, in the absence of rabies the average worm burden found in the population is much higher. The ecological reasoning behind this is increasing host longevity allowing more foxes to progress to higher worm burden categories. *E. multilocularis* is thus regulated by host availability and longevity, thus is indirectly regulated by rabies which directly suppresses host populations. This has broad implications for epidemiological models with multiple pathogens, as it is here demonstrated that the mode of transmission chosen for pathogens can have profound effects on how two pathogens are predicted to interact.

Given the physiological stress expected to be placed upon heavily parasitized individuals we further hypothesised that the most heavily parasitized individuals could experience a higher contact rate with other individuals due to a greater need to forage more widely; putting them at greater risk of contracting directly transmitted infections. We tested this by manipulating the rabies transmission coefficient, β_j, as functions of worm burden. Assuming that every worm works effects its host equally leads to a linear function of β_j. When this type of interaction is included in the model we observe a maximum 4.5% increase in the ability of rabies to suppress *E. multilocularis* populations when transmitted density dependently, and a minimum increase in *E. multilocularis* populations of 13% when transmitted frequency dependently. Suggesting that if a linear interaction exists and rabies is transmitted with frequency dependent dynamics that the interaction would actually benefit *E. multilocularis* populations and if transmission is density dependent there is only a small potential additional regulatory effect. Limitation of *E. multilocularis* biomass through host limitation by rabies virus has a much stronger, and thus more important, regulatory impact than heterogeneity in rabies transmission in both instances. The range of interaction strengths tested here range towards interactions so strong that they seem biologically unrealistic. As there are no data available to guide parameter choice, we extended the parameters to an extreme range in order to better understand our model behaviour. Weak interactions between rabies susceptibility and worm burden have the potential to be the most suppressive on *E. multilocularis* biomass and aggregation when rabies is modelled with density dependent transmission, and less suppressive on biomass as interaction strength increases when rabies is modelled with frequency dependent transmission. At high strength interactions, suppression of *E. multilocularis* biomass was weaker than in the absence of an interaction in all scenarios except for when rabies was transmitted with frequency dependent dynamics as an exponential function of worm burden, in which case increases in interaction strength caused mildly increased suppression. This is most likely due to the manner of the manipulation performed here: in order to make foxes with high worm burdens more susceptible to rabies, foxes with lower worm burdens have to be given a reduced susceptibility to rabies to hold R_0 constant. As only a very small minority of foxes are responsible for carrying the majority of worm biomass, at higher interaction strengths the majority of foxes have a much-reduced susceptibility to rabies,

which compensates for the minority of foxes with a very much-increased susceptibility to rabies. To test the implications of this assumption a sensitivity analysis between R_0 and interaction strength was performed. It is demonstrated that across a range of R_0 values for rabies that *E. multilocularis* is only weakly regulated by heterogeneity in rabies transmission. As increases in the R_0 of rabies have a strongly suppressive impact on fox density, this result is again indicative of *E. multilocularis* being most strongly supressed buy host availability and not heterogeneity in rabies transmission caused by over-dispersion of worms.

When β_j is modelled as function of worm burden, the minimum mean worm burden is up to 11.6% lower than when no interaction is included when rabies is modelled with density dependent transmission (both linear and exponential transmission functions) and a maximum of 23% lower than when no interaction is included under frequency dependent transmission (with an exponential transmission function). This suppression of mean worm burden is much greater than that seen in the total biomass of the population indicating that the most aggregated *E. multilocularis* infections are heavily supressed. This is an important result, as it shows that post-rabies eradication the worm burden of foxes may be predicted to increase, leading to *E. multilocularis* eggs being shed at higher rates per fox.

Due to the assumptions made here, quantitative predictions need to be interpreted with caution. Qualitatively however the results are important. The mechanism presented behind the hypothesis presented here is biologically sensible. An extremely heavily parasitized fox being more likely to contract rabies than a healthy fox is plausible given that we base this on an increased contact rate between individuals in a rabies endemic area. Realistically, the actual strength of this interaction is likely to depend on the carrying capacity and current density of the fox population in question: In an area of high fox density with small territory sizes a fox making extra-territorial excursions for food would likely contact several other foxes; conversely in a low density area with large territory sizes a fox making extra-territorial excursions would have a less increased contact rate. Here a wide range of interaction strengths are tested, and the outcomes from these differing strengths shown. Although the

model presented here cannot definitively quantify such an interaction without being confronted with epidemiological and ecological data, it certainly suggests this hypothesis is biologically plausible, and implicates the presence of endemic rabies as a suppressing force on *E. multilocularis* biomass.

As well as manipulating the strength of the interaction, two different forms of interaction were also tested. By distributing β_j exponentially we encompassed an interaction which affected foxes with extremely high worm burdens strongly while less strongly affecting those with low to moderate worm burdens. As infection with *E. multilocularis* is usually reported as being asymptomatic in the definitive host it is worthwhile testing the plausibility of an interaction in which the majority of infected foxes are relatively unaffected, and also to test the behaviour of the model in comparison to the linear interaction already used. There was little difference in the equilibrium biomass of *E. multilocularis*, densities of foxes or mean worm burdens obtained with these two functions of β_j, with very similar minimum values being reached at the same interaction strengths when rabies was modelled with density dependent transmission. Closer examination of *E. multilocularis* aggregation shows that an exponential relationship between β_j and worm burden has the potential to exert slightly stronger regulation on the distribution of worms amongst hosts in this scenario. Although the model, being theoretical in nature, is unable to prove the correct form of an interaction between these two pathogens in nature (if one indeed exists), it does serve to illustrate that competition for hosts between these two pathogens is more important in the regulation of *E. multilocularis* biomass and aggregation than an interaction between these two pathogens eliciting heterogeneity in rabies transmission. The two forms of interaction tested here differ only subtly when rabies is modelled with density dependent transmission. Importantly both forms of interaction demonstrate that a weak interaction between rabies and *E. multilocularis* (which in practice may be very difficult to detect) only has the potential to have a small additional regulatory effect on *E. multilocularis* populations. The elimination of rabies is thus predicted to facilitate population growth of this parasite by reducing the mortality rates of its definitive host. When this form of interaction was tested with rabies being transmitted frequency dependently there were again only small effects on total fox density and worm biomass, however the aggregation of worms amongst hosts

was more strongly affected under these conditions than any other tested, as worms and foxes in class W_4 tended towards zero, even at very low interaction strengths. As stated previously, recent work suggests that rabies may be more characteristic of a frequency dependent pathogen than a density dependent one, although where it lies on the scale between these two extremes in natural fox populations is currently not known. If however it is found to display frequency dependent transmission dynamics it may be the case that rabies is preventing extremely high *E. multilocularis* burdens from occurring in foxes. Thus the elimination of rabies may have potential to allow some individual foxes to acquire extremely high worm burdens, becoming "super-spreaders" of this zoonotic macro-parasite.

The model proposed here tests a variety of interaction strengths under both density and frequency dependent rabies transmission. This serves to show the flexibility this model may provide in being applied to other micro/macro-parasite systems, but also highlights the need for such a model to be confronted with real data. The worm burdens used in this model were based on worm aggregation reported in the presence of rabies. To better justify this model it should be confronted with data from populations where rabies has been eliminated to examine if the equilibrium population of *E. multilocularis* has indeed changed as the model predicts.

Whilst the success of rabies control strategies in Western Europe is certainly to be applauded, it would be foolish not to monitor the effects on other dangerous zoonoses post rabies elimination. Risks posed by rabies, both to human and wildlife health, are of global concern. Given this, every effort should be made to learn from current success stories, better equipping us to cope with the challenges faced. .

5 Plasticity in transmission strategies of the malaria parasite, Plasmodium chabaudi: environmental and genetic effects

5.1 Introduction

Parasites, like all sexually reproducing organisms, must optimize their resource allocation with respect to growth, survival and reproduction (Mideo & Reece 2012; Pollitt et al. 2011a). In the context of parasites, growth and survival within the host are synonymous with replication and reproduction synonymous with transmission to subsequent hosts (Koella & Antia 1995; Reece et al. 2009). The reproductive strategies of parasites are generally quantified in terms of R_0, the expected number of secondary infections arising from a single infected individual in a fully susceptible population (Anderson & May 1991a). However this singular epidemiological statistic does not capture heterogeneity in transmission, or document how parasites maximise their transmission potential with respect to the dynamic environment experienced during infections.

The allocation of resources to in-host survival and between-host transmission is a key fitness component for parasites and underpins the virulence and infectiousness of disease (Mideo & Reece 2012). For example, in *Plasmodium spp.*, the parasites responsible for causing malaria, survival in the host is maintained by cycles of asexual replication within red blood cells (RBC). A small proportion of the parasites produced every cell cycle differentiate into male and female sexual stages, termed gametocytes, which do not replicate in the host, but are required for transmission. We refer to this as the *proportional investment* in transmission. When taken up in a blood meal, gametocytes differentiate into gametes and fertilisation occurs. The requirement of different stages for within-host survival and between-host transmission makes *Plasmodium spp.* a powerful model to study the trade-off between survival and reproduction, an ecological and evolutionary concept traditionally studied in multicellular taxa (Roff 1992; Stearns 1992). Parasites investing heavily in gametocytes early in infections risk curtailing the duration for transmission due to insufficient asexual

replication to maintain the infection. Conversely, excessive investment in asexual replication reduces the rate of transmission and will also curtail duration if infections are so virulent the host dies. Broadly, however, *Plasmodium* invests remarkably little in transmission during infection of the mammalian host, with only a small proportion of merozoites undergoing gametocytogenesis with each round of asexual replication (Babiker et al. 2008; Dixon et al. 2008; Taylor & Read 1997). Several explanations have been proposed for this general restraint in its reproductive strategy. It may (i) reduce the virulence experienced by vectors by ensuring only a small number of gametocytes are taken up with any one blood meal (Cohuet et al. 2010); (ii) prevent hosts from developing gametocyte specific immunity, which would limit transmission (Buckling & Read 2001); or (iii) be an optimal strategy in the context of competition between co-infecting genotypes (Mideo & Day 2008), where most resources are invested in outcompeting conspecifics via asexual replication to ensure future transmission.

Plasmodium's search for an optimal transmission strategy is complicated by its dynamic host environment. During the acute phase of infection the host becomes progressively more anaemic as the parasite utilizes RBC to establish itself within the host. The subsequent infection dynamics are the result of the complex interplay between (i) the parasite's continued consumption of host resources, with potentially preferential tropism for certain ages of RBC and/or competition from co-infecting genotypes; (ii) the influx of immature reticulocytes as the result of the host's compensatory increase in erythropoiesis; (iii) innate immune responses, which may be associated with retention of RBC in the spleen and excessive inflammation resulting in the destruction of both infected and healthy RBC; and (iv) adaptive immune responses, likely directed against both shared and distinct antigens expressed by the asexual and sexual stages, and dynamically shifting focus in response to antigenic variation by the parasite (Day 2003; Koella & Antia 1995; Mideo et al. 2008; Paul et al. 2003).

Phenotypic plasticity is a ubiquitous evolutionary solution to the challenges of life in such a changing environment because it gives individual genotypes the ability to express the 'best' phenotype in response to its current environmental circumstances. *Plasmodium* indeed appears to exhibit such plasticity; investment in gametocytes varies with resource availability, drug treatment, the presence of other parasite genotypes, and host genotype (Buckling et al. 1999a;

Buckling et al. 1999b; Buckling et al. 1997; Hall et al. 2005; Pollitt et al. 2011b; Reece et al. 2010; Reece et al. 2005; Trager & Gill 1992; Trager et al. 1999). The sex ratio of gametocytes also varies during infections; erythropoiesis, anaemia (Paul et al. 2000), co-infection with conspecifics (Reece et al. 2008), low gametocyte density and transmission blocking immunity (Reece et al. 2008) increase investment in male relative to female gametocytes.

Is this observed plasticity truly adaptive? To address this question we first need to identify the environmental variables that influence the parasite's propensity to invest in transmission, and to what degrees. Understanding how parasites read cues from the host will then inform experimental tests of whether parasite transmission investment strategies are indeed optimal. However, theoretical and experimental studies have yielded conflicting conclusions in this area, particularly regarding the direction of changes in the proportional investment into gametocytes in response to changes in the in-host environment (Buckling et al. 1999a; Buckling et al. 1999b; Buckling et al. 1997; Pollitt et al. 2011a; Reece et al. 2010; Trager & Gill 1992; Wargo et al. 2007).

To better understand the extent to which environmental factors and parasite density shape transmission strategies, we use statistical models to explore how *P. chabaudi* invests in gametocytes as a function of parasite density and RBC availability in single infections of mice.

5.2 Methods

We quantify the influence of the host environment and parasite abundance on reproductive investment by the malaria parasite *Plasmodium chabaudi*. *P. chabaudi* exhibits a life cycle typical of all *Plasmodium spp*. (Figure 5.1): Merozoites are created when a single adult merozoite infects a host RBC where it develops through ring and trophozoite stages before asexually replicating into a schizont, which erupts 24 hours post-cell-invasion from the RBC releasing many more merozoites. Or alternatively, gametocytes are produced if a merozoite undergoes a longer 48 hour development in a RBC, where the ring stage develops straight into a mature gametocyte over this period.

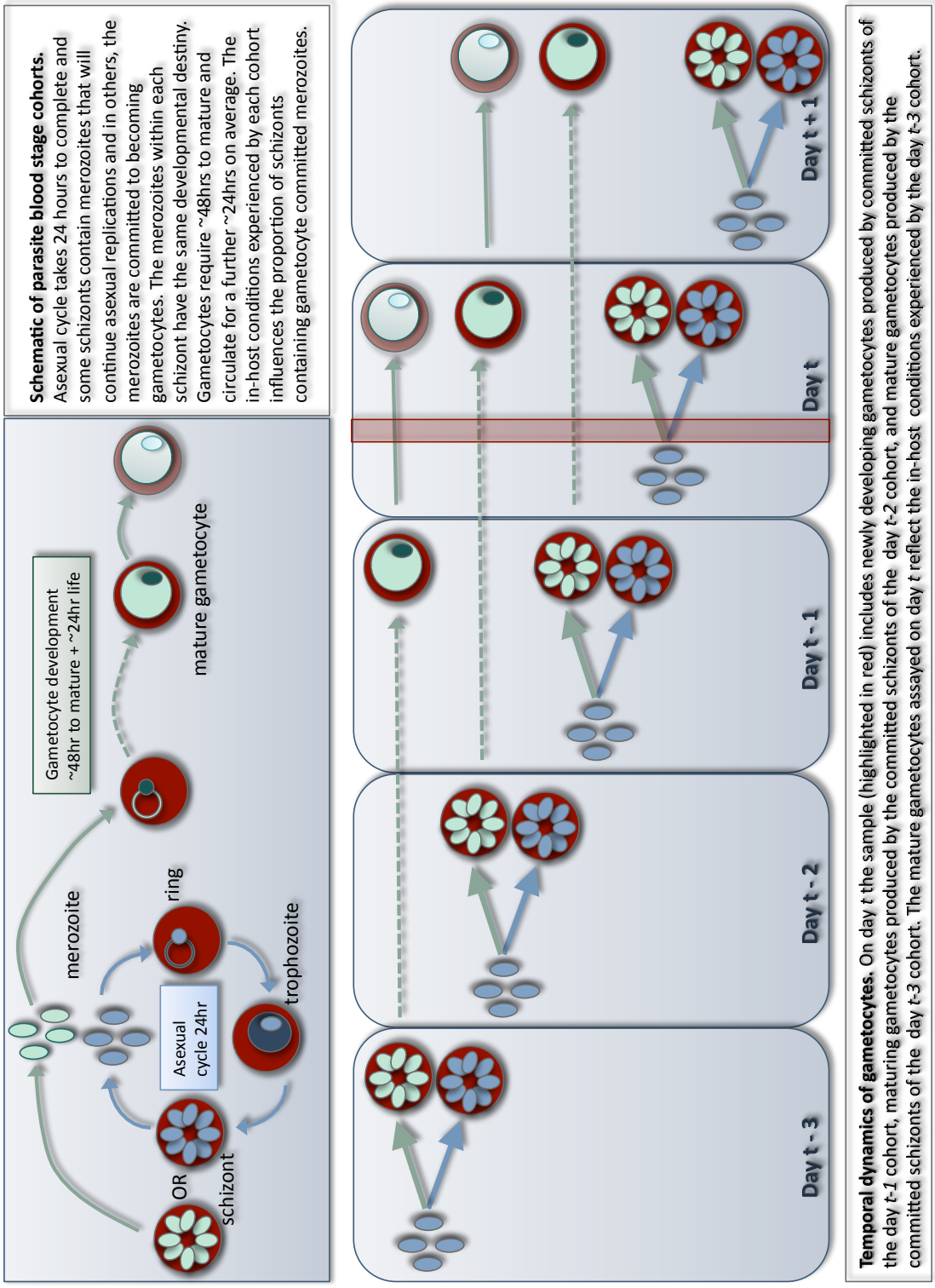

Figure 5.1 Schematic of asexual replication and gametocytogenesis in *P. chabaudi*. (Provided courtesy of Sarah Reece)

A uniquely large, previously published dataset (Pollitt et al. 2011b; Reece et al. 2008), from experimental infections of mice with six genetically distinct clones (hereafter referred to as genotypes) of the parasite is re-visited. Merozoites, the asexual life stage of the parasite, require 24 hours to mature in RBCs and gametocytes, the sexual life stage of the parasite, require 48 hours. Schizgony (eruption of infected RBCs) is synchronized and takes place every 24 hours post-infection (Buckling et al. 1999a; O'Donnell et al. 2011). This synchrony motivated the experimental sampling protocol and invites the use of discrete-time methods such as those used to model the dynamics of total parasite densities (Reece et al. 2008). For *Plasmodium*, the proportion of merozoites that differentiate into gametocytes, and the sex ratio of the gametocytes, are natural dynamic indicators of its allocation strategy. We predict gametocyte densities as functions of host or parasite factors at earlier time-points, accounting for the developmental delay between commitment to gametocytogenesis and observation of mature gametocytes. Using a minimum of modelling assumptions, we test whether the per-merozoite probability of differentiation at each replicative cycle, and the proportional investment in male gametocytes depend on the densities of parasites, mature and immature RBCs.

5.2.1 Experimental design

All experiments were carried out at the University of Edinburgh, UK. *P. chabaudi* genotypes from the WHO Registry of Standard Malaria Parasites were used. Infections described here were originally set up to examine sex ratios in single and mixed infections as described by Reece et al. (2008). Here we consider six single genotype infection groups (genotypes AJ, AS, ER, CR, CW, and DK) for analysis. In each treatment group five mice were inoculated with 1×10^6 parasites. Data are shown in Figure 5.2.

Mice used were 6-8 week-old MF1 males (in-house supplier, University of Edinburgh). All mice had blood sampled daily during the acute phase of infection; from day 5 post-infection (PI) until day 16 PI. Sampling took place in the morning to ensure circulating parasites were in ring or early trophozoite stages (and thus had not erupted from their host RBCs) and to ensure DNA replication for the production of daughter progeny had not yet occurred, as this would have confounded measurements taken by qPCR as additional genetic

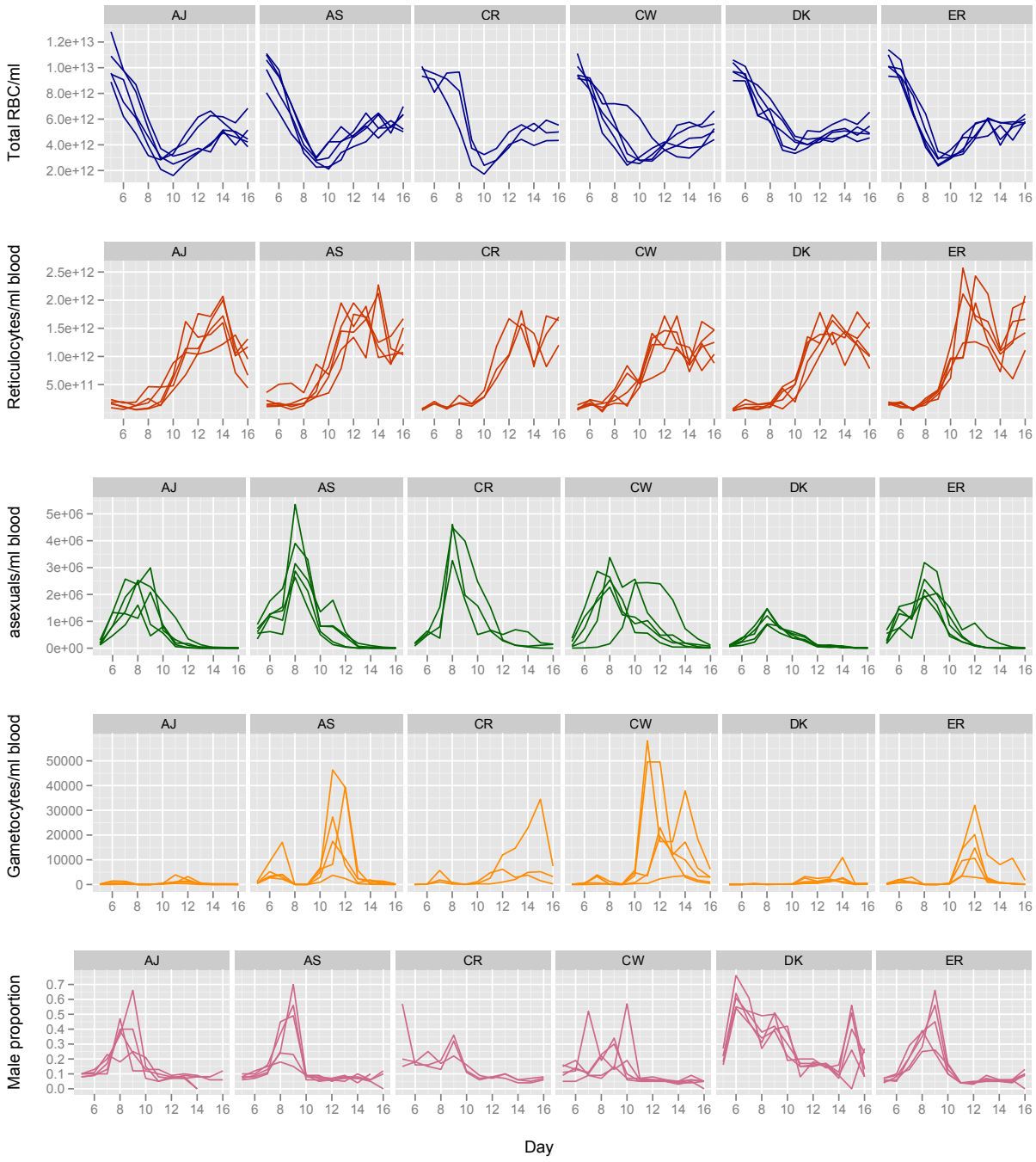

Figure 5.2 Time courses of RBC and parasite densities. Data from day 5-16 PI with six genotypes of *P. chabaudi* (AJ, AS, CR, CW, DK, ER). Longitudinal data, five mice per genotype (see Methods).

material from the next days generation would be present. Two mice from these treatment groups died before the end of this experimental period: Mouse 17 died at 10 days PI; Mouse 18 died at 12 days PI. Both mice were infected with genotype CR.

Polymerase chain reaction assays (Drew & Reece 2007) were used to distinguish and quantify asexual stages and gametocytes produced by each clone throughout

infection. Total RBC densities were estimated using flow cytometry (Coulter Counter, Beckman Coulter,(see Ferguson et al. 2003) and densities of immature RBCs (reticulocytes) were estimated from thin blood smears. All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

5.2.2 Models and analysis

We modelled gametocyte density (*G*) (parasites/ml of blood) and the proportion of circulating gametocytes that were male (*P*) as time-delayed functions of asexual density (*M*) and different ages of RBC. The delay is the interval between commitment to gametocytogenesis and the appearance of mature gametes in the circulation.

5.2.2.1 Modelling gametocytogenesis

We modelled gametocyte density *G* at day *t* in mouse *i* as a function of asexual density (*M*) *j* days previously (equation 5.1) or a combination of *M* and another host factor (equation 5.2);

$$G_{i,t} = \alpha_i M_{i,t-j}^{\beta_i} + e_{i,t} \qquad \textbf{Equation 5.1}$$

or

$$G_{i,t} = \alpha_i M_{i,t-j}^{\beta_i} \cdot X_{i,t-j}^{\gamma_i} + e_{i,t} \qquad \textbf{Equation 5.2}$$

where *X* was total red blood cells (RBC) (*T*) , reticulocytes (*R*), mature red blood cells (normocytes, *N*), or the proportion of gametocytes that were male (*P*). To normalize residuals, we log transformed the observables;

$$\log(G_{i,t}) = \log(\alpha_i) + \beta_i \log(M_{i,t-j}) + \varepsilon_{i,t} \qquad \textbf{Equation 5.3}$$

$$\log(G_{i,t}) = \log(\alpha_i) + \beta_i \log(M_{i,t-j}) + \gamma_i \log(X_{i,t-j}) + \varepsilon_{i,t} \qquad \textbf{Equation 5.4}$$

The residuals, $\varepsilon_{l,t}$, are assumed independent and drawn from $\mathrm{N}(0,\sigma^2)$, and initially the parameters α, β and γ were modelled to include mouse (*i*) as a normally distributed random effect.

Density dependence is modelled with the exponent β. If $G_{i,t} = \alpha M_{t-j}^{\beta}$, the *per capita* rate of conversion (the proportional investment in gametocytogenesis, or probability per merozoite of switching to the sexual stage) at day *t-j* is proportional to $\alpha M_{t-j}^{\beta-1}$. Thus β=1 in equation 5.1 implies that a constant proportion of merozoites convert to gametocytes during each replicative cycle with no adjustment in response to total parasite density. β>1 implies a positive correlation between the probability of switching and total parasite density, and β<1 a negative correlation. In the two-factor models, the quantity X^{γ} influences the conversion rate (the probability per parasite of differentiation into a gametocyte at each round of replication; synonymous with proportional investment) multiplicatively. When *X*=*R*, *T* or *N,* the exponent γ denotes the effect of resource availability; a value γ<0 means the parasite increases its proportional investment in gametocytes when resources decline. The constant of proportionality α includes the mortality of gametocytes between the initiation of their development and their observation.

5.2.2.2 Modelling allocation of transmission stages into male/female gametes

We assumed that the proportion of gametocytes that were male at day *t* in mouse *i*, $P_{i,t}$, was a function of a factor *X*, *j* days previously:

$$P_{i,t} = \alpha_i X_{i,t-j}^{\beta_i} + e_{i,t} \qquad \textbf{Equation 5.5}$$

or equivalently

$$\log(P_{i,t}) = \log(\alpha_i) + \beta_i \log(X_{i,t-j}) + \varepsilon_{it} \qquad \textbf{Equation 5.6}$$

where *X* is one of asexual density (*M*), gametocytes (G), total red blood cells (RBC) (*T*) , reticulocytes (*R*), or normocytes (*N*), and again the $\varepsilon_{l,t}$ are assumed independent and normally distributed with zero mean and equal variance.

5.2.2.3 Parameter estimation

Parameters were estimated using a linear mixed effects approach. The above models were fit to data for each genotype individually so as to avoid any

assumption that genotypes should be responding to their environment similarly. Zero values were assumed to be 0.5 of the smallest observed value of that covariate in the entire dataset. Model selection (using the Bayesian Information Criterion (BIC), with differences of 3 or greater considered significant) were robust to changes in this definition of the limit of detection. For gametocytogenesis, more complex models including additive contributions to *G* from multiple days were fitted using a nonlinear mixed effects approach and in all cases showed no significant improvement in fit over the simpler models (Equations 5.1 and 5.2). For the sex allocation model (5), multiplicative combinations of host factors analogous to (2) did not yield significantly better fits. For both models, analysis of different error structures indicated that only the intercept log(α) was required as a random effect. Time-lags of 2 or 3 days provided the best description of the data, with lags of 1 or 4 days yielding consistently poorer fits. Data were available from day 5 to 16 PI and so to allow comparison of models with different time-lags, all estimates were obtained using the gametocyte densities between days 8 and 16 PI. Analyses were performed in R version 2.14.0, using the lmer package (The R foundation for statistical computing; http://www.R-project.org)

5.3 Results

5.3.1 Proportional investment in gametocytes in influenced by both parasite and red blood cell density

Models of gametocytogenesis (equations 5.1 and 5.2) were fitted to each genotype separately as it has been demonstrated previously that they differ in their patterns of gametocytogenesis (Pollitt et al. 2011b). For all strains the best-fitting models were of the form of equation 5.2, with the multiplicative covariate *X* being either total red blood cells or reticulocytes, and with time-lags of 48 or 72 hours. (Table 5.1 and Figure 5.3).

Strain	Resource Sensitivity	Time Lag	Exponent β (Influence of parasite density)	Exponent γ (Influence of resource)
AJ	Total RBC	48h	0.61 (0.08)	-3.14 (0.58)
AS	Total RBC	72h	0.94 (0.14)	-3.40 (0.53)
CR	Reticulocytes	48h	0.76 (0.20)	1.70 (0.23)
CW	Reticulocytes	48h	0.49 (0.17)	1.60 (0.22)
DK	Total RBC	72h	0.92 (0.17)	-2.30 (0.50)
ER	Total RBC	72h	0.50 (0.13)	-4.50 (0.43)

Table 5.1 Summary of models of gametocytogenesis. The best-fitting models for all strains were of the form of equation 5.2, where *X* was total RBC or reticulocytes.

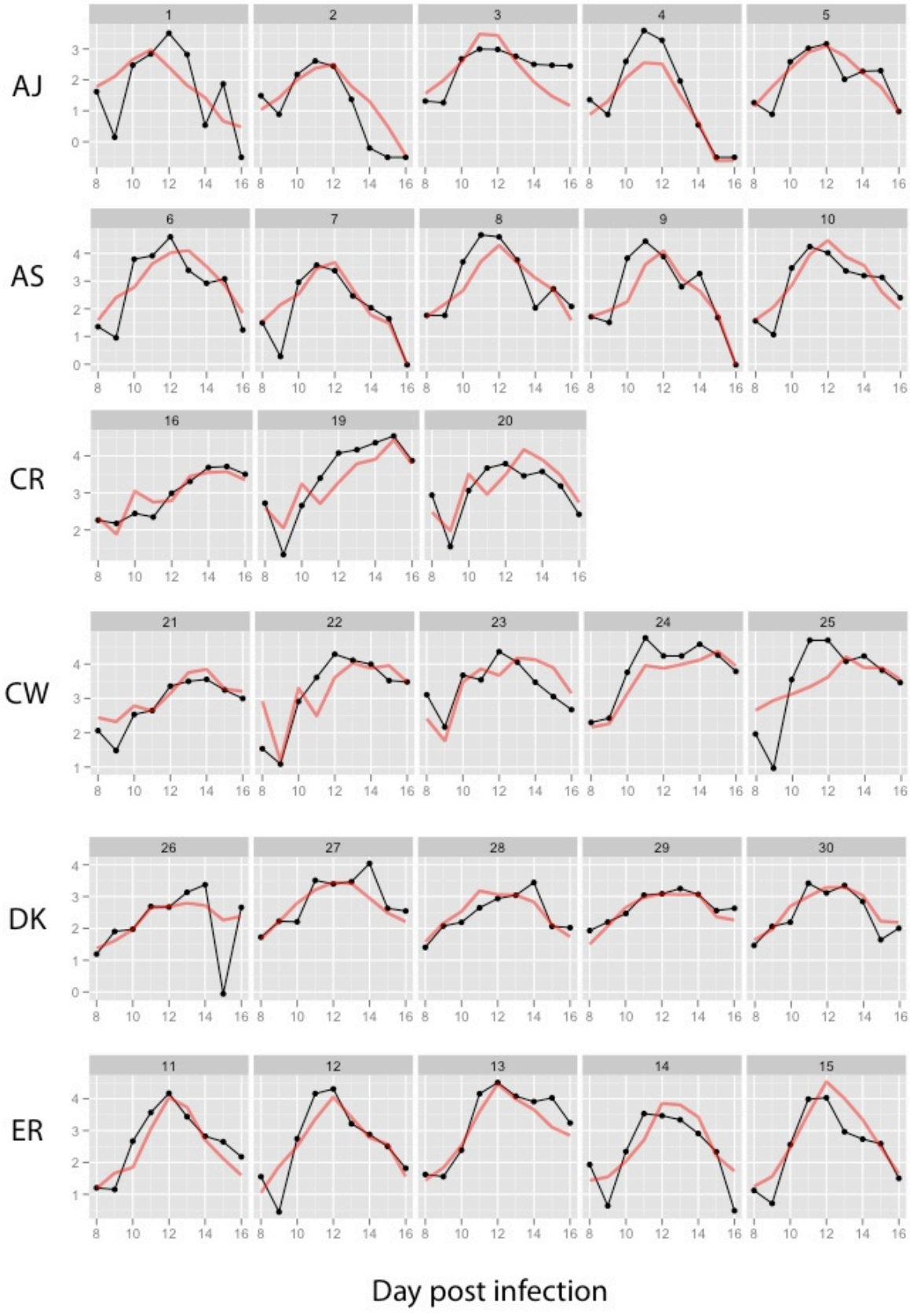

Figure 5.3 Best fits to gametocyte density for each mouse, by strain. Y axes show the logarithm to base 10 of gametocyte densities per ml of blood.

5.3.1.1 Dependence of conversion rates on parasite density

AS and DK had fitted exponents β close to unity (Table 1), suggesting no direct effect of parasite density on gametocyte investment in these genotypes. All other genotypes had $0.5 < \beta < 1$, showing that conversion rates were inversely but weakly related to parasite density; when RBC numbers are controlled for, we find no evidence for increased investment in transmission when parasites are abundant, and find that in most genotypes reproductive restraint is exercised when parasites are abundant.

5.3.1.2 Dependence on red blood cell counts

Four genotypes (AJ, AS, ER, DK) exhibited a strong negative correlation of conversion probabilities with total RBC numbers, delayed by 72h (AS, DK, ER) or 48h (AJ). Thus as the host becomes more anaemic, the more the parasite invests in transmission. In contrast, CR and CW showed a positive dependence on reticulocyte densities 48h previously.

5.3.1.3 Correlations between covariates

We found negative correlations between the logarithms of asexual parasite density (*M*) and total RBC in AJ, AS, CW and ER ($p<0.01$) and between asexuals and reticulocytes in all genotypes ($p<0.001$). To investigate the robustness of the dependencies established in Table 1, first we confirmed that for all genotypes, when comparing the estimate of the parameter β in the simpler model of equation 5.1 to that in equation 5.2 for each value of the time-lag *j*, the direction of the density-dependence was unchanged. That is, the 95% confidence intervals for β included unity for AS and DK, indicating no density-dependent conversion; and lay entirely below unity for AJ, CR, CW, ER. Similarly, we estimated the parameter γ in models of the form $G_t = \alpha M_t + X_{t-j}^{\gamma}$ - that is, forcing β=1 and modelling conversion as a function of X alone. Again, the density dependence remained, with strong negative correlation with total RBC at both 48h and 72h time-lags for AJ, AS,DK, ER ($\gamma<0$ for all strains, $p<0.001$) and a significant positive correlation with reticulocytes 48h previously for CR and CW ($\gamma<0$ $p<0.001$ for both strains).

5.3.1.4 Model validation and robustness; Testing the assumption of a constant probability of loss between commitment to gametocytogenesis and maturation

We used a single model and parameter set to describe gametocytogenesis over days 5-16 of infection, and assumed that a constant proportion of the parasites that commit to gametocytogenesis at day *t-j* survive to be observed at day *t*. This mortality rate is contained in the parameter α (equations 5.1 and 5.2). This analysis may be confounded by changes in the per-capita rate of loss of circulating gametocytes during the infection confound our analysis. For example, specific antibody responses develop slowly during the first two weeks of infection and may drive a progressive increase in the *per capita* rate of removal of gametocytes. We wanted to validate the assumption that, whatever changes in mortality may occur during infection, the same dependence of gametocytogenesis on the environmental parameters holds. To do this, for each strain we fitted models to five-day windows of data, first modelling gametocyte density on days 8-12 as functions of covariates on days 5-9, then on days 9-13 as functions of the covariates on days 6-10, and so on up to days 12-16 inclusive.

The analysis showed that (i) AJ and ER were consistently best described by their globally best-fitting models, with strongly negative dependence of conversion rates on total RBC; (ii) For windows up to days 10-14, CR and CW were described equivalently well by a positive dependence on reticulocytes lagged by 48h or a negative dependence on total RBC lagged by 72h, and by their global best fitting model (positive dependence on reticulocytes lagged by 48h) thereafter; (iii) until the last window of days 12-16, AS and DK were both described best by a 48h lagged RBC count with β <1, rather than the global best-fitting model of 72h-lagged total RBC count with β =1; and (iv) the parameter α declined with time for all strains except CR (approximate fractional decrease in α between intervals d8-d12 and d12-d16; AJ, 25%; AS, 20%; CW, 10%; DK, 8%; ER, 10%). This decline in α suggests that gametocyte mortality does increase during infection, most likely due to developing specific immune responses.

We can draw four conclusions here. First, irrespective of the time delay, for four strains we make the robust conclusion that throughout the observed course of infections, proportional investment in reproduction declines with RBC resources. Second, the variability in the time-lag (48h or 72h) suggests one or more of the

following are at play; variation in maturation time, gametocytes may survive in the circulation for longer than 24h, the possibility that merozoites pre-commit their progeny to gametocytogenesis or the assays employed detect gametocytes at slightly different maturation stages in different genotypes. Expression and morphology data demonstrate that transcription of assayed genes has occurred before gametocytes reach maturity, but the precise timing, and whether there is genetic variation for timing, are yet to be determined. Third, the variability in the exponent β suggests that dependence of reproductive investment on parasite densities is weak or may be confounded by other factors such as immune responses. Finally, over the course of infections with the two strains CR and CW, predictors of gametocytes moved between either a positive dependence on reticulocytes lagged 48h or negative dependence on total RBC lagged 72h. Consistent with these results, for these two strains only there were weak but significant negative correlations between reticulocytes and total RBC one day earlier (data not shown). Further, restricting the CR and CW analyses to days 5-13 yielded an overall negative dependence on total RBC, as for the other strains (delay of 72h; γ =-2.0 (CR) and γ = -4.1 (CW)).

5.3.2 Relative investment in male gametocytes over females is influenced weakly but positively by resource availability near the peak of acute infection

A negative correlation between investment in males and RBC availability was described for some genotypes previously (Reece et al. 2008). Indeed by inspection the proportion of male gametocytes increases between days 6 and 8 for most infections (Figure 5.2), suggesting that the worsening anaemia and increasing host stress up to day 5 or 6 post infection triggers increased investment in males, in line with theoretical predictions (Gardner et al. 2003; Ramiro et al. 2011). However, the present analysis models sex allocation in response to the host environment between days 5-13, with the response variable being the proportion of gametocytes that were male on days 7-15 (for a 48h correlation) or 8-16 (for 72h). We found that over days 8-16 PI, the proportion of gametocytes that were male was positively but weakly correlated with RBC numbers two or three days previously (Table 5.2). Further analysis showed that the correlation was apparent as the infection approached its peak, modelling *P* on days 8-12 as a function of covariates on days 5-9.

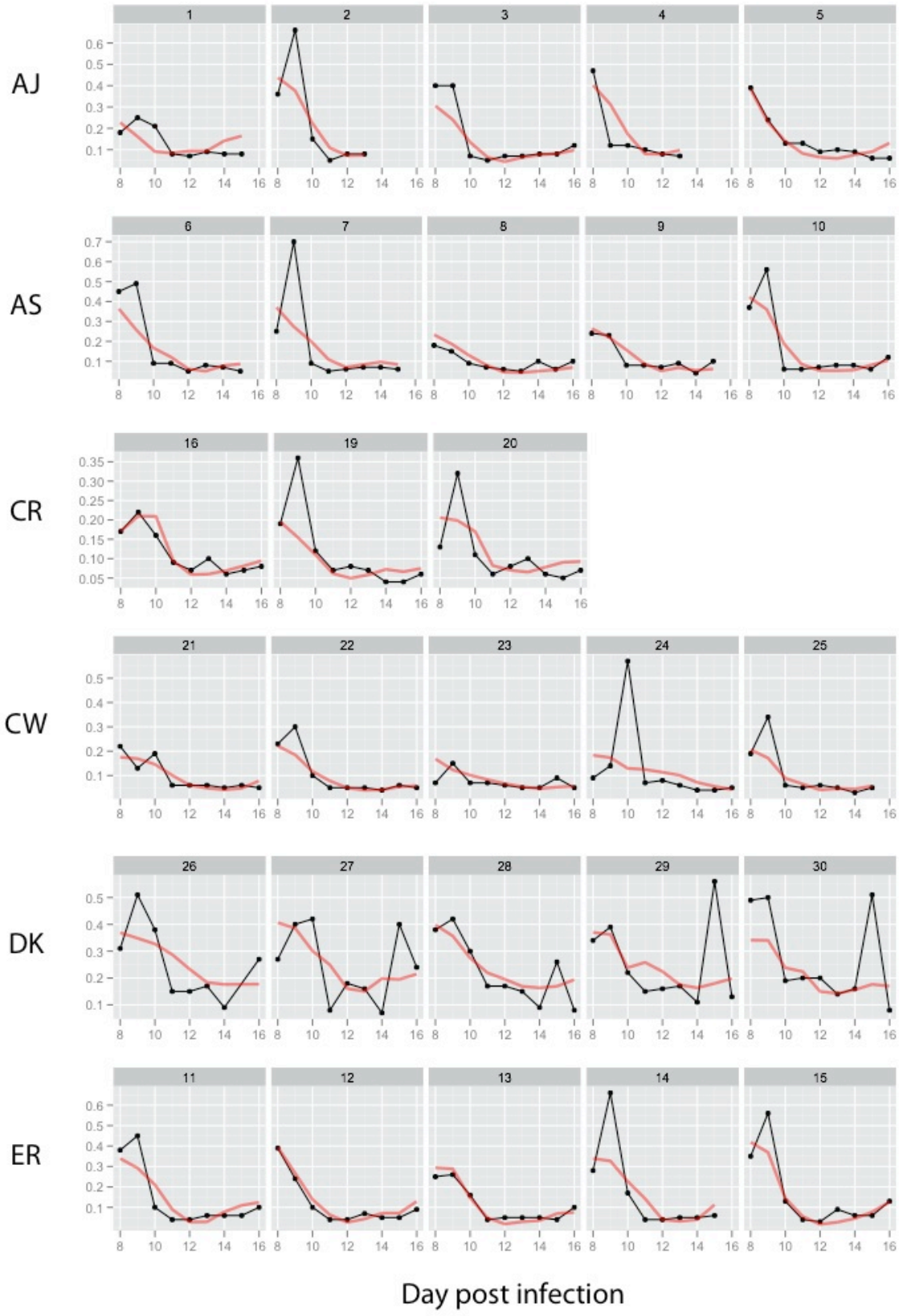

Figure 5.4 Best fits to male proportion for each mouse, by strain.

(Visually, this is apparent; proportional investment in males declines almost universally between days 9-12 and stays low thereafter. RBC also decline between days 6-9). From day 10 onwards, the degree of investment in males showed no correlation with any of the host factors we observed; that is, *P* on days 13-16 was independent of all covariates on days 10-13. Further, we saw no influence of parasite density, either asexuals or gametocytes, on the male:female ratio at any stage of infection with any genotype.

Strain	Best fit model indicates sensitivity to resource:	Time lag	Exponent β (standard error)
AJ	Normocytes	48h	0.19 (0.02)
AS	Normocytes	72h	0.17 (0.02)
	Total RBC	72h	0.24 (0.04)
CR	Normocytes	48h	0.11 (0.02)
CW	Normocytes	72h	0.11 (0.02)
	Total RBC	72h	0.15 (0.03)
DK	Total RBC	72h	0.21 (0.04)
	Total RBC	48h	0.25 (0.06)
	Normocytes	48h	0.16 (0.04)
	Normocytes	72h	0.13 (0.03)
ER	Total RBC	72h	0.29 (0.02)
	Normocytes	72h	0.19 (0.02)

Table 5.2 Modelling sex allocation across the entire infection. For some genotyopes, multiple models lay within range of 2 units of BIC and so were considered to have equivalent support.

5.4 Discussion

Our analyses of infections of rodents with six genotypes of *P. chabaudi* suggest that both RBC availability and asexual density influence the parasite's allocation of resources into reproduction. We find that investment in transmission increases rapidly as RBC numbers decline, and increases, albeit more weakly, at low parasite densities. We also find that the relative investment in males over females shows a shifting dependence on RBC availability. Finally, we validated the models by showing that the model that best described the whole time course of infection was also best fitting model within shorter temporal windows across days 5-16 post infection, and found evidence for increasing gametocyte mortality during this time frame.

In a previously published analysis of the same dataset (for which the main purpose was to look at the impact of competition on investment in gametocytes) conversion rates were found to correlate positively with the availability of total RBCs and the proportion that are reticulocytes for five of the six genotypes when in single infections (Pollitt et al. 2011b). The discrepancy between that study and this one may arise in part from the use of the formalism described in Buckling *et al*. (1999a) to relate asexual parasite densities and host factors to gametocyte densities at later times. In addition, we allow for possible nonlinearities in the dependence of proportional investment in gametocytes on parasite density and host factors, via the exponents β and γ (equations 5.1-5.4). There is no reason to assume that conversion rates are linearly related to RBC numbers, and indeed we identified strong nonlinear dependencies.

5.4.1 Mechanisms of sensing the environment

The mechanistic basis of this modulation of investment in reproduction is unclear. The probability of differentiation and relative investment in males is likely influenced by the host microenvironment; for example, soluble inflammatory mediators, or correlates of anaemia such as erythropoetin. Another non-exclusive possibility is that the parasite senses physical cues such as multiple infection of RBC; this could be a surrogate of high parasite densities and/or limited resources and thus means of quorum sensing.

5.4.2 Interpretation of parasite strategies

Since plasticity in gametocyte conversion rates was discovered (Carter & Miller 1979) the default evolutionary explanation has been an adaptation in which parasites demonstrate terminal investment in response to an emergency situation that threatens its survival within the host. At first sight our results seem to support this hypothesis – investment in transmission (terminal investment) increases as resources decline and when parasite numbers fall, both possible signifiers of a decrease in habitat quality. However, other interpretations of these relationships are possible. For example, (i) the development of host anaemia may correlate with the appearance of transmission-blocking immune factors in the blood. Increased investment in transmission may then compensate for the increased rate of death or damage to gametocytes, to maintain the probability of successful infection of the mosquito vector (Ramiro et al. 2011); or (ii) the development of host anaemia correlates to the presence or imminent appearance of reticulocytes. If the parasite can utilize this type of RBC, and in particular if reticulocytes support gametocyte development better than normocytes, rates of gametocytogenesis may increase with RBC loss. This may explain the positive correlation between conversion rates and reticulocytes for CR and CW.

Other observations question the emergency-transmission interpretation. We found that asexual density was either a weak negative influence on gametocyte investment (strains AJ, CR, CW, ER) or had no significant effect (AS, DK). Given that most malaria infections are not lethal, selection for terminal investment would seem more likely to be triggered by the threat of imminent clearance from the host, rather than anaemia, and so in contrast to our findings one might expect conversion to show a stronger dependence on asexual densities than on environmental variables. Also, under stressful, competitive (co-infection) conditions, parasites appear to prioritise their in-host survival over short-term transmission (Pollitt et al. 2011a; Pollitt et al. 2011b; Reece et al. 2010). Thus in light of our results and these recent studies the evidence supporting the terminal investment hypothesis is underwhelming.

5.4.3 Correlates of virulence

The six genetically distinct genotypes used here vary in their level of virulence. Given the trade-off parasites experience between transmission and virulence (Alizon & van Baalen 2008; Bull 1994; Day 2003) it may be expected that genotypes exhibiting similar levels of virulence might modify their investment in transmission in response to the same environmental cues. Indeed we found the strength of the dependence of the rate of gametocytogenesis on parasite density does correlate with virulence. The two avirulent strains (AS and DK) show the lowest sensitivity of reproduction to asexual density (β closest to unity), and the most virulent genotypes (AJ, CW, ER) show the highest (β furthest from unity). In contrast, sensitivity to RBC availability did not correlate with virulence. DK, AJ, AS and ER showed increasingly strong dependence on total RBC densities. These genotypes exhibit a wide range of virulence, from weakly (AS, DK) to moderately (AJ) to highly virulent (ER) (Bell et al. 2006; Mackinnon et al. 2005; Mackinnon & Read 2003). Similarly the two strains most sensitive to changes in reticulocyte densities exhibit different levels of virulence, with CW being more virulent than CR (Bell et al. 2006; Taylor & Read 1997; Taylor et al. 1997).

The harm these strains do to their hosts is positively correlated with their competitive ability in mixed infections (Bell et al. 2006). Thus competitive fitness may be related to the ability to more strongly modulate investment in transmission in response to parasite densities, and to commit most strongly to asexual growth when parasites are at low densities, outcompeting co-infecting strains. Indeed a pathogen's immunogenicity and the virulence deriving from the associated immunopathology may be related more strongly to rates of change in pathogen density than the pathogen burden itself (Hatta et al. 2010). The genetic variation for these reaction norms suggests that genotypes may divergently evolve and have the potential to respond to selection pressures.

5.4.4 Top down vs. bottom up control

Pressures on parasites within hosts are often partitioned conceptually into top-down (immune-mediated) or bottom-up (resource limitation) forces (Graham 2008; Haydon et al. 2003). Without readouts of immune responses or inflammatory markers, we cannot discount the possibility that the RBC

covariates in our models are also correlates of immune responses; it is possible that rather than responding to resource availability the parasite is altering its strategy in response to immunity. The distinction between the immune-and resource-mediated pressures may also be a blurry one, given that excessive immune responses, particular relatively indiscriminate responses by splenic macrophages, may give rise to extensive lysis of uninfected RBC and loss of parasite resources (Evans et al. 2006; Schofield & Grau 2005). Thus bystander damage and extensive parasite replication mean that early in the infection, RBC loss and immune responses may be positively correlated. Indeed destruction of bystander target cells has been proposed to be an adaptive host strategy, creating a fire-break that limits pathogen growth (Handel et al. 2009). As anaemia worsens within the host the parasite may also have to invest more in overcoming transmission blocking immune factors, and both innate and adaptive immune responses to the parasite will limit transmission (Carter et al. 1979).

5.4.5 The timing of developmental cues

We found comparable support for models that explained gametocyte densities as functions of the asexual densities and host environment 48 and 72h previously. Current understanding of the *P. chabaudi* life cycle is gametocytes take 48h to develop following infection of a red blood cell. However, the period during which *Plasmodium* commits to the sexual stage, or how this decision can be influenced, is unclear. All *P. falciparum* parasites within one infected cell result from a single developmental choice, suggesting that initiation of differentiation happens before replication within a red blood cell (Silvestrini et al. 2000; Smith et al. 2000). Further, it has been suggested that merozoites may be developmentally pre-committed to differentiation, imprinted with cues received by the parental parasites (Dixon et al. 2008), allowing for the possibility of a 72h delay between triggering of differentiation and the appearance of mature gametocytes. Alternatively, if mature gametocytes remain in circulation sufficiently long, circulating gametocyte densities may be superpositions of two cohorts that committed 2 and 3 days previously. However we found no significant improvement in fit using models that modelled gametocytes as a weighted sum of contributions from two cohorts separated by 24h.

5.4.6 Altering sex allocation in response to RBC availability

The sex ratio of gametocytes circulating in the host bloodstream changes over the course of infection. Male gametocytes are thought to suffer more than females from agglutination by antibodies, as this inhibits the motility of their gametes necessary for fertilization (reviewed in West et al. 2001). *Plasmodium spp.* appear to adaptively adjust sex ratio in response to the inbreeding rate, which is determined by the genetic composition of infections. This involves producing female biased sex ratios in clonal infections to minimize competition for mates between related male gametocytes, and increasing investment in males in mixed infections to better compete for fertilizations (Hamilton 1967; West et al. 2001). Parasites are also predicted to increase the production of male gametocytes to compensate for both scarcity of gametocytes and increased male-biased predation by the host immune system (Gardner et al. 2003; Reece et al. 2008). By inspection of the data, early in infection investment in males does increase as RBC numbers decline, triggered perhaps in response to circulating levels of erythropoetin (Paul et al. 2000). However we find that this relation reverses as anaemia worsens and identify a positive correlation between sex ratio and RBC numbers over d5-13 post-infection; subsequent to d13, sex allocation shows no correlation with any host or parasite factors. It seems likely that any facultative adjustment of sex ratio in response to bottom-up control becomes masked at later time-points by response to top-down immune factors and potentially also differential survival of male and female gametocytes in the face of increasingly intense, specific immune responses (Reece et al. 2003).

Explaining plasticity in transmission by parasites as terminal investment in response to a threatening environment is certainly appealing in its simplicity. Remarkably, there has been little work carried out to quantify “emergency situations” posed by a parasite’s changing environment or to verify that terminal investment sufficiently increases short-term transmission potential to warrant this strategy. Given that the presence of a superior competitor is not enough to induce emergency transmission (Pollitt et al. 2011b) it would be difficult to justify that clones in a single infection, as is the case here, are facing an emergency. By applying a novel method of assessing the environmental cues used by *P. chabaudi* for determining investment in transmission we find support for recent studies that question terminal investment. We propose hypotheses to

explain our result that investment in transmission in clonal infections is modified in response to changes in resource availability and, to a lesser extent, parasite density. Ultimately, additional experiments are required to fully disentangle the complex mechanisms behind, and fitness consequences of plastic transmission strategies of *P. chabaudi*. Future work should focus on the effects of parasite age preference for RBC and the role of host immunity in determining transmission effort. Experiments chemically manipulating RBC densities (for example with EPO or phenylhydrazine), and manipulations of host immunity (through vaccination, drugs and infective doses) will undoubtedly prove important next steps in furthering our understanding of the ecological and evolutionary processes underlying parasite transmission strategies.

6 General discussion

Epidemiology has its foundations built upon assumptions of homogeneity: hosts homogenously distributed in space, homogenous in their infectiousness and susceptibility and homogenous in their rate of contacting other individuals. The work presented in this thesis covers a broad spectrum of topics, and demonstrates the wide range of ecological factors that can elicit heterogeneity in disease transmission. Studying disease transmission in an ecological context will be necessary to better understand the role of transmission heterogeneity in disease emergence, epidemic dynamics, and to better evaluate the impact disease eradication has on pathogen communities. Exploring epidemiology in the context of the ecology faced by pathogens has become a very active area of research over the past decade, with consideration being given to the dynamic environment pathogens experience within their hosts (Haydon et al. 2003; Pedersen & Fenton 2007; Reece et al. 2010); the community dynamics of pathogens (Cox 2001; Graham 2008; Pedersen & Fenton 2007); the role of host community demography, social interactions and spatial structure play in disease spread and persistence (Beyer et al. 2011; Craft et al. 2008; Haydon et al. 2002; Lloyd-Smith et al. 2005). The effect ecology has on the epidemiological dynamics of pathogens is well recognised for its potential significance, but in reality, the field of disease ecology is still very much in its infancy. Taking a cross-disciplinary approach, it is shown that transmission may vary in response to ecological changes occurring at different environmental scales.

Empirical evidence for density dependent disease transmission is examined in Chapter 2. Despite debate on how host population density should be incorporated into epidemiological models (De Jong et al. 1995; McCallum et al. 2001), there is remarkably little evidence for a simple relationship between host density and transmission rate. Experimentally, density dependent transmission can be demonstrated, but is not well explained by the traditional Anderson and May formulations (described in Anderson & May 1991b). Experimental populations can be controlled to reasonably resemble the assumptions made by theoretical models; specifically density can be held as close to homogenous with carefully chosen host species and experimental set-up.

In naturally occurring host populations density is not homogenous, and the risk faced by an individual to contract a density dependently transmitted disease will be affected by the local population density, and not necessarily the global density. The local density experienced by an individual will vary due to the species in question, its behaviour and social structure/interactions. It may be the case that pathogen transmission is best described by density dependent functions at local scales (within a social group, such as a pack), and frequency dependent on a global scale (between social groups). This could be responsible for the complex dynamics observed in measles epidemics; whereby high densities of children in schools lead to local epidemics, but infectious contacts between schools result in dynamics better explained by frequency dependence. If this is the case, then metapopulation models incorporating aspects of both density and frequency dependent transmission should better describe epidemics in large populations than models that ignore heterogeneity in density and transmission. Transmission functions used in traditional epidemiological models, although intuitively sensible, are an over-simplification of a dynamic and variable process. The development of more sophisticated epidemiological models will be reliant on developing new data driven approaches, which allow for breaks in assumptions of homogeneity.

A component community was described in Chapter 3, with an aim to examine co-infection relationships. Studies of disease community at this scale have typically included only two pathogen species, or two types of pathogen — for example skin parasite, or intestinal parasite (Balestrieri et al. 2006; Hamilton et al. 2005; Harbison et al. 2008; Jones et al. 2008). We utilised a variety of techniques to test for a wide scope of diseases. This allowed for a more comprehensive examination of a naturally occurring component community than has been done previously.

There was evidence for one pair of pathogens occurring together more often than expected by chance alone, and no evidence of pathogens occurring together less frequently than expected by chance, which would have been indicative of competitive exclusion. Interestingly, the two pathogens involved in a co-infection relationship were both micro-parasites: the intracellular apicomplexan *Toxoplasma gondii*, and the virus, canine adenovirus type-I (CAV-I). This makes the trade-off between Th1 and Th2 immune responses

experienced by the host an unlikely mechanism behind this relationship, but still possible if hosts were also infected with a macro-parasite that was not tested for here. Hosts suffering from this co-infection were however of significantly lower condition than other hosts sampled. It could be that differences in the quality of hosts lead to some hosts being more prone to co-infection if they have fewer resources to devote to their immune system, but equally it may be that this specific combination of pathogens is detrimental to the health of the host. The data collected here do not allow for testing the direction of this relationship, but this does provide an avenue for future experimental work. If it is the case that co-infections were more common in poorer quality hosts, it may be expected that ecological changes that lower host quality (for example habitat degradation, introduction of alien competitors) could facilitate the spread of disease and interfere with component community dynamics.

Care should be taken in interpreting the results from this fox study. Whilst providing a more comprehensive description of a component community than many previous studies, it does suffer from small sample size issues, making it difficult to extrapolate these results beyond those individuals sampled. Studies with larger sample sizes are needed to confront the results presented here. There is a general lack of statistical power for detecting co-infection relationships, so it is also possible that relationships between the diseases tested for were simply too weak to be detected. However, this research is suggestive that co-infection relationships should be expected to occur in natural host populations and that individual differences in the quality of hosts may be related to co-infection. Importantly, this study also provides a framework for utilising cross-disciplinary methods for studying disease communities.

The regulatory impact of a micro-parasite on a macro-parasite is explored theoretically in Chapter 4. These are both zoonotic diseases of public health concern. This is done in the context of rabies eradication in Western Europe and the subsequent spread of the small fox tapeworm *Echinococcus multilocularis*. These two pathogens utilise different transmission strategies that may be considered at opposite ends of a continuum: Rabies is highly pathogenic with extremely efficient transmission over a short infectious period before killing the host, while *E. multilocularis* is asymptomatic, and is transmitted rather inefficiently over a sustained period of time without killing its host. Rabies thus

has the potential to limit *E. multilocularis* populations through limiting available hosts, and removing any co-infecting *E. multilocularis*.

A model was developed to compare the total environmental load, and aggregation of *E. multilocularis* in the presence and absence of rabies. It was found that in the absence of rabies the total *E. multilocularis* burden of the host population is higher, and more aggregated. This has implications for the efficacy of disease eradication programmes: In this instance, eradication of one dangerous zoonosis has resulted in an increase of another, and an increase in the proportion of hosts that can act as "super spreaders". We also tested if rabies could further supress *E. multilocularis* populations if the aggregation of worms caused heterogeneity in rabies transmission — such that those hosts with the highest worm burdens were most at risk from rabies infection. Low levels of heterogeneity in rabies transmission caused a small additional suppression of *E. multilocularis* populations, with higher levels of heterogeneity reducing the suppressive effect of rabies. Although somewhat counter-intuitive, this result highlights that interactions between pathogens may act with variable and unexpected outcomes that are, in practice, impossible to predict. Disease eradication programmes are likely to become more common, given the current threats posed by emerging and re-emerging disease. As for any natural community, human induced extinction can have undesirable effects; it may even be expected that some pathogens will be analogous to "keystone species" in traditional community ecology, and removal of these pathogens will have the most profound effects on pathogen community stability. Disease surveillance needs to become an integral part of disease eradication programmes; ideally prior to, during and post-disease control efforts.

The effect of changes in the within-host environment on the transmission strategies of parasites was examined in Chapter 5. Six genetically distinct clones of the rodent malaria parasite *Plasmodium chabaudi* are shown to exhibit plasticity in how much they invest in transmission in response to changes in their immediate environment during the acute phase of infection. We find evidence that *P. chabaudi* increases its investment in transmission in response to decreasing resource availability, and a weaker effect of increase in parasite density causing a decrease in transmission investment which correlates with virulence of the genotypes.

This study shows heterogeneity in transmission may arise from two sources: 1) environmental conditions experienced within the host and 2) genetic variability between parasites. Precisely identifying the causal mechanisms behind this heterogeneity is not possible with this dataset, and ultimately additional experimental work manipulating resource availability and host immunity will be needed to fully disentangle the processes at play. Nevertheless, the results represent an important step in identifying the adaptive nature of transmission strategies employed by this parasite. Previous work has suggested that parasites should utilise terminal investment strategies when environmental factors threaten the persistence of infection. Our results add to a growing body of literature that suggests that optimal parasite transmission strategies are not as simplistic as this.

The plasticity in transmission strategies identified in Chapter 5 again has implications concerning heterogeneity in disease transmission. Using *P. chabaudi* as a model pathogen, we demonstrate that pathogens may work to maximise a trade-off between maintaining infection and maximising transmission in response to a changing environment. In nature, host quality will be much more variable than in the laboratory set-up utilised here, which has the potential to lead to extremely variable transmission rates between individual hosts. Variation in within host habitats will be further contributed to by concomitant infections (Cox 2001; Pollitt et al. 2011b), previous exposure and immunity (Buckling & Read 2001) and drug treatment regimes (Buckling et al. 1999a; Buckling et al. 1999b). Understanding how, and why parasites adapt their transmission strategies in response to their environment is necessary to consider bigger issues in disease ecology and evolution: how parasites may be evolving to change their virulence, transmission rates, drug resistance, and even how they evolve the ability to transmit to new species. Only by understanding the evolutionary trade-offs faced by pathogens can we begin to tease apart the mechanisms responsible for the changing threats posed by emerging disease.

Through the course of this thesis I have strived to use cross-disciplinary methods to study a range of ecological aspects affecting disease transmission. These studies provide a useful insight into some of the ecological dynamics that affect transmission, and demonstrate the importance of considering factors that may affect transmission at different ecological scales. It is shown that there is a

general need to relax the assumptions of homogeneity, which are commonplace in epidemiological studies. Information on heterogeneity in pathogen – host systems is frequently sparse, but by building upon current epidemiological models, there is no reason that the expected impacts of heterogeneity in transmission should not be further explored. It is shown here that more information is needed on the effects of disease eradication on pathogen communities. Future work should focus on monitoring disease in populations following the successful control of the pathogen of interest. Future empirical studies should also take measures of individual host differences that may be important in predicting the likelihood of animals becoming "super-spreaders" for disease. There is a great need to better understand how the immune status of hosts affects the ability of a pathogen to transmit, and the strategy it employs to maximise transmission potential. Understanding heterogeneity in transmission will benefit the design of successful disease control programmes, and be important in monitoring their success. In todays globally changing environment understanding the ecology affecting the spread of disease is of the upmost importance, and necessary to understanding the transmission dynamics responsible for disease emergence, and effectively evaluating the threats disease posed to endangered wildlife. Perhaps most importantly, this thesis documents the different scales on which transmission is influenced by ecology, and highlights the need to consider these different scales if we are to improve our abilities to control disease, predict epidemics and perhaps even predict disease emergence. Future work will hinge on collaborative efforts between researchers working in mathematics, ecology, microbiology, immunology and molecular biology (at the very least) to make maximum progress on this work.

7 Appendices

7.1 R code for Chapter 4

/Users/guscameron85/Documents/R/rabwormappend.r Page 1 of 6
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```r
##############################################################################
## Rabworm contains all functions of beta ##
##############################################################################
# 30/05/2012
contour.data=data.frame(NULL)
# Load libraries

library(deSolve)
library(odesolve)

# Define models

#=============================================================================================
nullfunc = function(t,y,params){
    with(as.list(params),{

    dW0.dt= ((a*sum(y))*((K-sum(y))/K)) - (b*y[1]) -
            ((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
            (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
            (beta*y[1]*y[6])

    dW1.dt=((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
            (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
            (b*y[2]) - (sigma1*y[2]) - (beta*y[2]*y[6])

    dW2.dt=(sigma1*y[2]) - (b*y[3]) - (sigma2*y[3]) - (beta*y[3]*y[6])

    dW3.dt=(sigma2*y[3]) - (b*y[4]) - (sigma3*y[4]) - (beta*y[4]*y[6])

    dW4.dt=(sigma3*y[4]) - (b*y[5]) - (beta*y[5]*y[6])

    dI.dt=(beta*y[1]*y[6]) + (beta*y[2]*y[6]) +
            (beta*y[3]*y[6]) + (beta*y[4]*y[6]) +
            (beta*y[5]*y[6]) - (b*y[6]) - (gamma*y[6])


    return(list(c(dW0.dt, dW1.dt, dW2.dt, dW3.dt, dW4.dt, dI.dt)))
    })
}
#=============================================================================================

#=============================================================================================
denslinear = function(t,y,params){
    with(as.list(params),{

    dW0.dt= ((a*sum(y))*((K-sum(y))/K)) - (b*y[1]) -
            ((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
            (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
            ((x*(1+(i0/z)))*y[1]*y[6])

    dW1.dt=((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
            (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
            (b*y[2]) - (sigma1*y[2]) - ((x*(1+(i1/z)))*y[2]*y[6])

    dW2.dt=(sigma1*y[2]) - (b*y[3]) - (sigma2*y[3]) - ((x*(1+(i2/z)))*y[3]*y[6])

```

```
57      dW3.dt=(sigma2*y[3]) - (b*y[4]) - (sigma3*y[4]) - ((x*(1+(i3/z)))*y[4]*y[6])
58
59      dW4.dt=(sigma3*y[4]) - (b*y[5]) - ((x*(1+(i4/z)))*y[5]*y[6])
60
61      dI.dt=((x*(1+(i0/z)))*y[1]*y[6]) + ((x*(1+(i1/z)))*y[2]*y[6]) +
62              ((x*(1+(i2/z)))*y[3]*y[6]) + ((x*(1+(i3/z)))*y[4]*y[6]) +
63              ((x*(1+(i4/z)))*y[5]*y[6]) - (b*y[6]) - (gamma*y[6])
64
65
66      return(list(c(dW0.dt, dW1.dt, dW2.dt, dW3.dt, dW4.dt, dI.dt)))
67
68      })
69  }
70  #=============================================================================================
71
72  densexponential = function(t,y,params){
73      with(as.list(params),{
74
75      dW0.dt= ((a*sum(y))*((K-sum(y))/K)) - (b*y[1]) -
76              ((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
77              (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
78              ((x*(1+(exp(0)/z)))*y[1]*y[6])
79
80      dW1.dt=((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
81              (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
82              (b*y[2]) - (sigma1*y[2]) - ((x*(1+(exp(i1*c)/z)))*y[2]*y[6])
83
84      dW2.dt=(sigma1*y[2]) - (b*y[3]) - (sigma2*y[3]) - ((x*(1+(exp(i2*c)/z)))*y[3]*y[6])
85
86      dW3.dt=(sigma2*y[3]) - (b*y[4]) - (sigma3*y[4]) - ((x*(1+(exp(i3*c)/z)))*y[4]*y[6])
87
88      dW4.dt=(sigma3*y[4]) - (b*y[5]) - ((x*(1+(exp(i4*c)/z)))*y[5]*y[6])
89
90      dI.dt=((x*(1+(exp(0)/z)))*y[1]*y[6]) + ((x*(1+(exp(i1*c)/z)))*y[2]*y[6]) +
91              ((x*(1+(exp(i2*c)/z)))*y[3]*y[6]) + ((x*(1+(exp(i3*c)/z)))*y[4]*y[6]) +
92              ((x*(1+(exp(i4*c)/z)))*y[5]*y[6]) - (b*y[6]) - (gamma*y[6])
93
94
95      return(list(c(dW0.dt, dW1.dt, dW2.dt, dW3.dt, dW4.dt, dI.dt)))
96      })
97  }
98  #=============================================================================================
99
100 freqlinear = function(t,y,params){
101     with(as.list(params),{
102
103     dW0.dt= ((a*sum(y))*((K-sum(y))/K)) - (b*y[1]) -
104             ((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
105             (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
106             (((x*(1+(i0/z)))*y[1]*y[6])/(y[1]+y[6]))
107
108     dW1.dt=((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
109             (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
110             (b*y[2]) - (sigma1*y[2]) - (((x*(1+(i1/z)))*y[2]*y[6])/(y[2]+y[6]))
111
112     dW2.dt=(sigma1*y[2]) - (b*y[3]) - (sigma2*y[3]) -
```

```
113             (((x*(1+(i2/z)))*y[3]*y[6])/(y[3]+y[6]))
114
115     dW3.dt=(sigma2*y[3]) - (b*y[4]) - (sigma3*y[4]) -
116             (((x*(1+(i3/z)))*y[4]*y[6])/(y[4]+y[6]))
117
118     dW4.dt=(sigma3*y[4]) - (b*y[5]) - (((x*(1+(i4/z)))*y[5]*y[6])/(y[5]+y[6]))
119
120     dI.dt=(((x*(1+(i0/z)))*y[1]*y[6])/(y[1]+y[6])) +
121             (((x*(1+(i1/z)))*y[2]*y[6])/(y[2]+y[6])) +
122             (((x*(1+(i2/z)))*y[3]*y[6])/(y[3]+y[6])) +
123             (((x*(1+(i3/z)))*y[4]*y[6])/(y[4]+y[6])) +
124             (((x*(1+(i4/z)))*y[5]*y[6])/(y[5]+y[6])) - (b*y[6]) - (gamma*y[6])
125
126
127     return(list(c(dW0.dt, dW1.dt, dW2.dt, dW3.dt, dW4.dt, dI.dt)))
128
129     })
130 }
131 #=============================================================================
132
133 freqexponential = function(t,y,params){
134     with(as.list(params),{
135
136     dW0.dt= ((a*sum(y))*((K-sum(y))/K)) - (b*y[1]) -
137             ((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
138             (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
139             (((x*(1+(exp(0)/z)))*y[1]*y[6])/(y[1]+y[6]))
140
141     dW1.dt=((lambda*((y[2]*i1)+(y[3]*i2)+(y[4]*i3)+(y[5]*i4))*
142             (sum(y)-y[1]-y[6]))/(Ho+(sum(y)-y[1]-y[6])))*(y[1]/(sum(y)-y[6])) -
143             (b*y[2]) - (sigma1*y[2]) - (((x*(1+(exp(i1*c)/z)))*y[2]*y[6])/(y[2]+y[6]))
144
145     dW2.dt=(sigma1*y[2]) - (b*y[3]) - (sigma2*y[3]) -
146             (((x*(1+(exp(i2*c)/z)))*y[3]*y[6])/(y[3]+y[6]))
147
148     dW3.dt=(sigma2*y[3]) - (b*y[4]) - (sigma3*y[4]) -
149             (((x*(1+(exp(i3*c)/z)))*y[4]*y[6])/(y[4]+y[6]))
150
151     dW4.dt=(sigma3*y[4]) - (b*y[5]) - (((x*(1+(exp(i4*c)/z)))*y[5]*y[6])/(y[5]+y[6]))
152
153     dI.dt=(((x*(1+(exp(0)/z)))*y[1]*y[6])/(y[1]+y[6])) +
154             (((x*(1+(exp(i1*c)/z)))*y[2]*y[6])/(y[2]+y[6])) +
155             (((x*(1+(exp(i2*c)/z)))*y[3]*y[6])/(y[3]+y[6])) +
156             (((x*(1+(exp(i3*c)/z)))*y[4]*y[6])/(y[4]+y[6])) +
157             (((x*(1+(exp(i4*c)/z)))*y[5]*y[6])/(y[5]+y[6])) - (b*y[6]) - (gamma*y[6])
158
159
160     return(list(c(dW0.dt, dW1.dt, dW2.dt, dW3.dt, dW4.dt, dI.dt)))
161     })
162 }
163 #=============================================================================
164
165
166 #Data frames for storing outputs
167 worm.data = data.frame(NULL)
168 fox.data = data.frame(NULL)
```

```
169 total.data= data.frame(NULL)
170
171 #for (R0 in seq(from=1, to=1.75, by=0.25)){
172 for (strength in 0:20){
173 #for(R0 in seq(from=1, to=2.75, by=0.25)){
174 # Worm burdens and fox densities at equilibrium in the absence of rabies:
175 i0=0
176 i1=20
177 i2=427
178 i3=3221
179 i4=32309
180 W0 = 0.1307111
181 W1 = 2.8711044
182 W2 = 1.7485746
183 W3 = 1.4584499
184 W4 = 0.4814256
185 c=1e-3
186
187 # Manipulation of Beta
188
189 strength = strength
190 R0 = 1.4
191 b = 0.002
192 Gamma = 0.14
193 z = i4/(strength)
194
195 #====== Manipulations of beta heterogeneity ======#
196 # Comment out as appropriate:
197
198 #denslinear:
199 x = (R0*(b+Gamma))/(W0+(W1*(1+(i1/z)))+(W2*(1+(i2/z)))+(W3*(1+(i3/z)))
200      + (W4*(1+(i4/z)))) #linear
201 betar = c( x*(1+(i0/z)),x*(1+(i1/z)),x*(1+(i2/z)),x*(1+(i3/z)),x*(1+(i4/z)) ) # Linear
202 R0check =( (betar[1]*W0) + (betar[2]*W1) + (betar[3]*W2) + (betar[4]*W3) +
203         (betar[5]*W4) ) / (b+Gamma) # Linear
204 print(R0check)
205
206 # densexponential:
207 #x = (R0*(b+Gamma))/(W0*(1+(exp(0*c)/z))+(W1*(1+(exp(i1*c)/z)))+(W2*(1+(exp(i2*c)/z)))+
208 #    (W3*(1+(exp(i3*c)/z))) + (W4*(1+(exp(i4*c)/z)))) # exponential
209 #betar = c( x*(1+(exp(i0*c)/z)),x*(1+(exp(i1*c)/z)),x*(1+(exp(i2*c)/z)),
210 #        x*(1+(exp(i3*c)/z)),x*(1+(exp(i4*c)/z)) )
211 #R0check =( (betar[1]*W0) + (betar[2]*W1) + (betar[3]*W2) + (betar[4]*W3) +
212 #            (betar[5]*W4) ) / (b+Gamma)
213 #print(R0check)
214
215 # freqlinear:
216 #x = (R0*(b+Gamma)) / ((1+((i0)/z)) + (1+((i1)/z)) + (1+((i2)/z)) +
217 #    (1+((i3)/z)) + (1+((i4)/z)))
218 #betar= c( x*(1+((i0)/z)), x*(1+((i1)/z)), x*(1+((i2)/z)), x*(1+((i3)/z)), x*(1+((i4)/z)
219 #R0check =( (betar[1]) + (betar[2]) + (betar[3]) + (betar[4]) + (betar[5]) ) / (b+Gamma)
220 #print(R0check)
221
222 # freqexponential:
223 #x = (R0*(b+Gamma)) / ((1+(exp(0*c)/z)) + (1+(exp(i1*c)/z)) + (1+(exp(i2*c)/z))
224 #     + (1+(exp(i3*c)/z)) + (1+(exp(i4*c)/z)))
```

```
225 #betar = c( x*(1+(exp(0*c)/z)), x*(1+(exp(i1*c)/z)), x*(1+(exp(i2*c)/z)),
226 #       x*(1+(exp(i3*c)/z)), x*(1+(exp(i4*c)/z)) )
227 #R0check =( (betar[1]) + (betar[2]) + (betar[3]) + (betar[4]) + (betar[5]) ) / (b+Gamma)
228 #print(R0check)
229
230
231 # Model Parameters
232
233 params = (list(a = 0.00452,
234             b = 0.002,
235             K = 12,
236             lambda = 42,
237             Ho = 3259.89,
238             gamma = 0.14,
239             i0=0,
240             i1=20,
241             i2=427,
242             i3=3221,
243             i4=32309,
244             sigma1=2.7879e-3,
245             sigma2=2.8421e-3,
246             sigma3=5.7143e-4,
247             beta=0.02971,
248             c=1e-3
249         ))
250
251
252 # Initial conditions
253
254 y0 = c(W0=5, W1=1, W2= 0, W3=0, W4=0, I=1)
255 t = 1:10000
256
257 # Solve; return data.frame, "out"
258 func = denslinear # declare model to solve
259 out = ode(y0,t,func,params) #daspk
260
261 # Organise and save model outputs:
262
263 out = data.frame(out)
264 out$H = out$W0+out$W1+out$W2+out$W3+out$W4 +out$I
265 out$P = (out$W1*i1)+(out$W2*i2)+(out$W3*i3)+(out$W4*i4)
266
267 total.run = data.frame( strength=strength,
268                 Fox = out$H[out$time==max(out$time)],
269                 Worm = out$P[out$time==max(out$time)],
270                 Burden = out$P[out$time==max(out$time)]/out$H[out$time==max(out$time)],
271                 Rabies = out$I[out$time==max(out$time)])
272
273
274 fox.run = data.frame(   Strength=strength, W0=out$W0[out$time==max(out$time)]/total.run$
275                 W1=out$W1[out$time==max(out$time)]/total.run$Fox,
276                 W2=out$W2[out$time==max(out$time)]/total.run$Fox,
277                 W3=out$W3[out$time==max(out$time)]/total.run$Fox,
278                 W4=out$W4[out$time==max(out$time)]/total.run$Fox)
279
280 worm.run = data.frame(  Strength=strength, P0 = 0,
```

```
281                 P1=((out$W1[out$time==max(out$time)]*i1)/total.run$Worm),
282                 P2=((out$W2[out$time==max(out$time)]*i2)/total.run$Worm),
283                 P3=((out$W3[out$time==max(out$time)]*i3)/total.run$Worm),
284                 P4=((out$W4[out$time==max(out$time)])*i4)/total.run$Worm)
285
286 contour.run = data.frame(R0=R0, Strength=strength, Worm=total.run$Worm, Fox=total.run$Fo
287 contour.data=rbind(contour.data, contour.run)
288
289
290 fox.data=rbind(fox.data,fox.run)
291 worm.data=rbind(worm.data, worm.run)
292 total.data=rbind(total.data, total.run)
293
294 # change file names as appropriate:
295
296 write.csv(total.data, "densexponentialtotal.csv", row.names=FALSE)
297 #write.csv(fox.data, "denslinearfox.csv", row.names=FALSE)
298 #write.csv(worm.data, "denslinearworm.csv", row.names=FALSE)
299 #write.csv(contour.data, "freqexponentialcontour.csv", row.names=FALSE)
300
301 } # end for loop
302 #} # end R0 loop
303
```

7.2 R code for Chapter 5


```
1  ##############################################
2  ## Anaysis with new data transformations     ##
3  ##############################################
4
5  #LOAD LIBRARIES
6  library(FME)
7  library(nlme)
8  library(deSolve)
9  library(splines)
10 library(ggplot2)
11 library(lattice)
12
13 #LOAD AND ARRANGE OBSERVED DATA
14 gus=T
15
16 if(gus) setwd("~/Documents/R")
17 else setwd("~/Documents/Projects/Collaborations/SarahReece/Autumn2010/")
18
19 data=read.csv("malaria_1b.csv", header=T)
20 data=subset(data, !is.na(RBC_count))
21 source("Malaria-Function-defs6b.R") # New Function defs which us M^2
22
23 ###################################
24 # BASE FUNCTIONS
25 ###################################
26
27 # X shall need defined for each nlsList.
28
29 f1= function(beta, X) beta*X
30 f2= function(beta1, beta2, X.1, X.2) beta1*X.1 + beta2*X.2
31 f3= function(beta1, beta2, beta3, X.1, X.2, X.3) beta1*X.1 + beta2*X.2 + beta3*X.3
32
33
34 ###############################
35 ###############################
36 #_________ANALYSIS_________
37 ###############################
38 ###############################
39
40 g="AJ" # Enter genotype name here
41
42 # Arrange neccessary data:
43 d=process.data(data, genotype=g)
44 d=groupedData(Gam~ Day|Mouse, data=d)
45
46 #_____________f1_____________
47 fit1=nlsList(Gam~f1(beta, X=X1) | Mouse,
48                 data=d, start=list(beta=2))
49 s1=nlme(fit1)
50
51 fit2=nlsList(Gam~f1(beta, X=X2) | Mouse,
52                 data=d, start=list(beta=8))
53 s2=nlme(fit2)
54
55 fit3=nlsList(Gam~f1(beta, X=X3) | Mouse,
56                 data=d, start=list(beta=3))
57 s3=nlme(fit3)
```

```
 58
 59 fit4=nlsList(Gam~f1(beta, X=X4) | Mouse,
 60                 data=d, start=list(beta=1))
 61 s4=nlme(fit4)
 62
 63 fit5=nlsList(Gam~f1(beta, X=X5) | Mouse,
 64                 data=d, start=list(beta=0.5))
 65 s5=nlme(fit5)
 66
 67 fit6=nlsList(Gam~f1(beta, X=X6) | Mouse,
 68                 data=d, start=list(beta=1))
 69 s6=nlme(fit6)
 70
 71 fit7=nlsList(Gam~f1(beta, X=X7) | Mouse,
 72                 data=d, start=list(beta=1))
 73 s7=nlme(fit7)
 74
 75 fit8=nlsList(Gam~f1(beta, X=X8) | Mouse,
 76                 data=d, start=list(beta=0.2))
 77 s8=nlme(fit8)
 78
 79 fit9=nlsList(Gam~f1(beta, X=X9) | Mouse,
 80                 data=d, start=list(beta=0.44))
 81 s9=nlme(fit9)
 82
 83 fit10=nlsList(Gam~f1(beta, X=X10) | Mouse,
 84                 data=d, start=list(beta=0.45))
 85 s10=nlme(fit10)
 86
 87 fit11=nlsList(Gam~f1(beta, X=X11) | Mouse,
 88                 data=d, start=list(beta=1))
 89 s11=nlme(fit11)
 90
 91 fit12=nlsList(Gam~f1(beta, X=X12) | Mouse,
 92                 data=d, start=list(beta=0.3))
 93 s12=nlme(fit12)
 94
 95 fit13=nlsList(Gam~f1(beta, X=X13) | Mouse,
 96                 data=d, start=list(beta=18))
 97 s13=nlme(fit13)
 98
 99 fit14=nlsList(Gam~f1(beta, X=X14) | Mouse,
100                 data=d, start=list(beta=34))
101 s14=nlme(fit14)
102
103 fit15=nlsList(Gam~f1(beta, X=X15) | Mouse,
104                 data=d, start=list(beta=16))
105 s15=nlme(fit15)
106
107 fit16=nlsList(Gam~f1(beta, X=X16) | Mouse,
108                 data=d, start=list(beta=0.5))
109 s16=nlme(fit16)
110
111 fit17=nlsList(Gam~f1(beta, X=X17) | Mouse,
112                 data=d, start=list(beta=0.612))
113 s17=nlme(fit17)
114
```

```
fit18=nlsList(Gam~f1(beta, X=X18) | Mouse,
                data=d, start=list(beta=0.33))
s18=nlme(fit18)

fit19=nlsList(Gam~f1(beta, X=X19) | Mouse,
                data=d, start=list(beta=1))
s19=nlme(fit19)

fit20=nlsList(Gam~f1(beta, X=X20) | Mouse,
                data=d, start=list(beta=0.3))
s20=nlme(fit20)

fit21=nlsList(Gam~f1(beta, X=X21) | Mouse,
                data=d, start=list(beta=0.5))
s21=nlme(fit21)

fit22=nlsList(Gam~f1(beta, X=X22) | Mouse,
                data=d, start=list(beta=0.46))
s22=nlme(fit22)

fit23=nlsList(Gam~f1(beta, X=X23) | Mouse,
                data=d, start=list(beta=0.3))
s23=nlme(fit23)

fit24=nlsList(Gam~f1(beta, X=X24) | Mouse,
                data=d, start=list(beta=0.46))
s24=nlme(fit24)
#____________________________________

#_________________f2_________________
fit25=nlsList(Gam~f2(beta1, beta2, X.1=X1, X.2=X2) | Mouse,
                data=d, start=list(beta1=0.3, beta2=3))
s25=nlme(fit25)

fit26=nlsList(Gam~f2(beta1, beta2, X.1=X4, X.2=X5) | Mouse,
                data=d, start=list(beta1=1, beta2=1))
s26=nlme(fit26)

fit27=nlsList(Gam~f2(beta1, beta2, X.1=X7, X.2=X8) | Mouse,
                data=d, start=list(beta1=1, beta2=1))
s27=nlme(fit26) # Dummy Fit !!!

fit28=nlsList(Gam~f2(beta1, beta2, X.1=X10, X.2=X11) | Mouse,
                data=d, start=list(beta1=1, beta2=1))
s28=nlme(fit26) # Dummy Fit !!!

fit29=nlsList(Gam~f2(beta1, beta2, X.1=X13, X.2=X14) | Mouse,
                data=d, start=list(beta1=0.4, beta2=12.4))
s29=nlme(fit29)

fit30=nlsList(Gam~f2(beta1, beta2, X.1=X16, X.2=X17) | Mouse,
                data=d, start=list(beta1=1, beta2=1))
s30=nlme(fit30)

fit31=nlsList(Gam~f2(beta1, beta2, X.1=X19, X.2=X20) | Mouse,
                data=d, start=list(beta1=1, beta2=1))
s31=nlme(fit31)
```

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