Life-history correlates of *Mycobacterium bovis* infection in individual Eurasian badgers (*Meles meles*)

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Abstract

Bovine tuberculosis caused by *Mycobacterium bovis* is a disease of global importance. In the UK, it has serious economic and welfare implications for cattle farming enterprises, and its control confers substantial costs on UK taxpayers. Disease control strategies, in particular those pertaining to its main wildlife reservoir, the Eurasian badger (*Meles meles*), are highly problematic and continue to divide opinion. The aim of the present study was to investigate life-history correlates of infection with *M. bovis* in individual badgers, using data from the long-term badger trapping and sampling research programme at Woodchester Park in south-west England. An understanding of disease manifestations at the individual level is essential to elucidate transmission dynamics at the social group and population levels, and is therefore also important in the development and optimisation of disease control strategies.

Epidemiological analyses centre on the correct interpretation of diagnostic test results. In the case of bovine tuberculosis, this is hindered by the complexity and variability of the immune response. Recent data from the Woodchester Park population presented a rare opportunity to observe the temporal progression of the cell-mediated response as measured by the gamma-interferon assay in a population of free-living naturally infected badgers. Analysis demonstrated fluctuation and decline in the interferon response over time following initial detection. In addition, the magnitude of the initial response was positively correlated with the likelihood of disease progression. These data provide a useful framework on which to further our understanding of the pathogenesis of naturally acquired *M. bovis* infection in badgers.

Using retrospective data collected over a 24 year period, condition loss was shown to be a feature of disease in badgers, but only when mycobacterial excretion was detected. Furthermore, adult female badgers appeared to show more resilience to the physiological impact of disease than male badgers, as they survived for longer, gained weight as per the normal seasonal cycle, and continued to reproduce successfully despite intermittently excreting *M. bovis*. Shorter survival times were also reported for badgers in which the onset of excretion was characterised by positive culture from a bite wound or lymph node abscess.

A more intensive study of six badger social groups in the study area over three years from 2007 to 2010 revealed no significant association between the magnitude of the IFN response and either the presence or intensity of helminth or coccidial burdens in individual badgers, providing no evidence to support a simple relationship between parasite burdens and the immune response to *M. bovis* infection.

The ability of infectious adult females to continue to reproduce and rear cubs successfully resulted in significantly higher risks of both the acquisition and progression of infection in cubs captured in the same social group. In addition, the highest probability of pre-emergent infection was observed in cubs from these high-risk groups. There was a decreasing risk gradient observed from the infectious breeding female to seropositive breeding females to other adults of excretor then seropositive status, and there was no evidence to support a protective effect of maternally derived antibody in cubs. However, groups with infectious breeding females were in the minority during the study period from 1982 to 2010, and the majority of emergent cubs in the population were not detected as infected during their first year of life. These findings highlight the importance of social structure and the role of infectious females in disease dynamics.

The value of potential control strategies such as the targeted selective culling of seropositive adult females, and annual vaccine delivery to as many cubs as possible prior to infection, either solely or in combination, are discussed in the light of these findings.

Declaration of authorship

I, Alexandra Jane Tomlinson, declare that this thesis is my own work. I have acknowledged all the main sources of help, and all specific sources of information.

Alexandra Jane Tomlinson

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CHAPTER ONE: General Introduction

1.1 Historical background of bovine tuberculosis in cattle and wildlife in the UK

Bovine tuberculosis is caused by *Mycobacterium bovis*, a member of the *M. tuberculosis* complex (Karlson and Lessel 1970). The pathogen is capable of infecting a very wide range of mammal hosts including humans (Hardie & Watson 1992, de Lisle *et al.* 2001).

Bovine tuberculosis is a disease of worldwide importance both from an economic and a human health point of view. It poses a considerable economic problem for cattle farming enterprises in the UK (Bourne *et al.* 2007), Ireland (Olea-Popelka *et al.* 2003), New Zealand (Coleman & Cooke 2001), and Spain (Naranjo *et al.* 2008) and is also currently an increasing threat to free living wildlife in the Kruger and Hluhluwe Umfolozi National Parks in South Africa (Renwick *et al.* 2007, Michel *et al.* 2009). In 2008/2009 the cost of tuberculosis in cattle to the UK Government, including compensation, control measures and research was over £108 million (Defra 2011a).

In the early twentieth century, bovine tuberculosis was a significant cause of disease in humans in the United Kingdom (Hardie & Watson 1992). A combination of milk pasteurisation, starting in 1930, and a compulsory cattle test and slaughter control policy in 1950 reduced disease in the human and cattle populations. However, infection in cattle persisted in some areas, particularly in south-west England (Muirhead *et al.* 1974), raising suspicions that a wildlife reservoir could be involved. A high prevalence of infection of *M. bovis* in badgers examined *post-mortem* was found in the vicinity of infected farms in south-west England (Muirhead *et al.* 1974), leading to the implementation of several badger culling strategies. However, in 1997, following a Government review (Krebs *et al.* 1997), culling was suspended pending the outcome of the randomised badger culling trial (RBCT) designed, and overseen, by the Independent Scientific Group (ISG). This experiment compared the cattle herd breakdown rates in trial areas subjected to one of three treatments; no culling, reactive culling on land where cattle TB breakdowns were confirmed, and proactive culling across the whole area. The results of the trial identified both negative and positive effects of badger culling (Bourne *et al.* 2007). Reactive culling on land where TB breakdowns had been confirmed was associated with a significant increase in the incidence of cattle herd breakdowns and this aspect of the trial was in fact suspended in 2003 (Donnelly *et al.* 2003). Proactive culling was found to significantly reduce

the incidence of cattle herd breakdowns in the proactive area, but with an associated significant increase in cattle herd breakdowns in the surrounding area. This was attributed to perturbation of the badger population (Woodroffe *et al.* 2006, Carter *et al.* 2007). The final report of the ISG was unequivocal in recommending that a badger culling strategy in the UK was unlikely to provide a meaningful contribution to reducing the incidence of new cattle herd breakdowns, due to the modest benefits, high cost and potential detrimental effects. At the time, however, these conclusions were questioned in a report by the UK Government's Chief Scientific Adviser (King 2007). Publications subsequent to the cessation of the trial have continued to emerge, reporting that negative effects on the incidence of cattle herd breakdowns associated with perturbation of badger populations appear to reduce over time, but any net gains inside the proactive areas remain modest, and high financial costs and potential negative effects remain a significant problem (Donnelly *et al.* 2007, Jenkins *et al.* 2008a, Jenkins *et al.* 2010). Badger culling remains a subject of great controversy with polarisation of the views of interested parties. The most recent policy development in England has been an announcement of intent to pursue an industry-funded licensed badger culling strategy (Defra 2011b). There is no doubt that the controversy will continue and the implementation of future policy options remains unpredictable at best.

Recently badger vaccination has emerged as a potential long-term control strategy to reduce the incidence of cattle herd breakdowns. Injectable Bacille Calmette-Guérin (BCG) has been licensed for use in badgers (VLA/FERA 2010), and is currently being deployed in several areas of England, funded by Government in the form of the Badger Vaccine Deployment Project in Gloucestershire (Defra 2011c), by Gloucestershire Wildlife Trust on their reserves (Gloucestershire Wildlife Trust 2011), and by the National Trust on one of their estates in Devon (National Trust 2011). Research is ongoing with respect to development and optimal deployment strategies for an oral bait for badgers containing BCG vaccine.

1.2 Badger ecology

Badgers are generally social animals, living in groups and occupying underground burrow systems (setts), but levels of sociality and territorial behaviour vary with population density. At higher densities, such as those found in much of south-west England (Neal & Cheeseman 1996), a relatively rigid social structure may exist, with clear boundary demarcation and little territorial overlap (Rogers *et al.* 2000). Territory boundaries are demarcated by the use of latrine areas, with social group members carrying out frequent faecal and glandular scent marking (Kruuk 1978). At low population densities, social

structure is more flexible, with greater territory overlap and little boundary demarcation (Cresswell & Harris 1988).

Badgers are nocturnal omnivores, and in the UK, exhibit a particular preference for earthworms (particularly *Lumbricus spp.*), but insects, small mammals, cereals and fruits are also taken (Kruuk & Parish 1981). Badger body condition fluctuates throughout the year, peaking in the autumn and falling during the winter associated with a reduction in activity (Kruuk & Parish 1983). The wooded, hilly landscape of the Cotswolds is ideal badger habitat and as a result very high densities have been recorded there (Rogers *et al.* 2000).

Badgers breed annually, with the birth of most cubs in mid-February in Great Britain. Cubs remain below ground in the sett until they emerge no earlier than about eight weeks after birth (Neal & Cheeseman 1996). Mating behaviour has two peaks, one in spring and one in the late summer - autumn (Cresswell *et al.* 1992). Fertilisation takes place at the same time as mating, although implantation of the blastocyst is delayed until the autumn, when it is triggered by reducing daylength and an individual's body condition (Woodroffe 1995).

1.3 The immunopathogenesis of mycobacterial infections

The manifestation of disease associated with *M. bovis* infection is highly variable both within and between host species, and arises as a consequence of a complex interaction between the host and the pathogen (Thorns & Morris 1983, Flynn & Chan 2001, van Crevel *et al.* 2002). Information relating to the immunopathogenesis of mycobacterial infections is scarce in many species, particularly wildlife.

Pathogenic mycobacteria are intracellular organisms. As such, successful control and/or elimination by the host are predominantly driven by the adaptive immune response which in turn, is likely to be characterised by a strong cell-mediated component (Janeway *et al.* 2005). Central to the adaptive immune response are the lymphocytes, (a type of white blood cell), which are categorised by their differing effector functions. B lymphocytes (B cells) produce antibodies and represent the humoral arm of the adaptive immune response. T lymphocytes (T cells) represent the cell-mediated arm of the adaptive immune response. The nature of the type of immune response is determined by the interaction between the host and the pathogen and is a key determinant of the outcome following infection (Fenton *et al.* 2008). T-helper (Th) cells are critical in modifying the immune response in accordance with the pathogenesis of the invading pathogen. Cell-mediated responses are associated with Th_1 cells and humoral responses with Th_2 cells.

The majority of mycobacterial infections are acquired by the respiratory route (Orme 2004). Following inhalation, alveolar macrophages act to ingest and destroy bacilli and may prevent infection from establishing (Lurie & Dannenberg 1965). Bacillus virulence and host macrophage competence influence this process. Mycobacteria which survive then proceed to multiply within macrophages, disrupting their function and triggering an acute local granulomatous inflammatory response. The subsequent non-specific release of cytokines such as gamma-interferon (IFN) and tumour necrosis factor (TNFα) further activate macrophages (Walzl *et al.* 2011). Once again, control (and/or possible elimination) at this stage will depend on the balance between macrophage success and bacillus resistance mechanisms. The adaptive immune response is fully triggered within 2-3 weeks following infection, through antigen presentation by macrophages and dendritic cells. This usually results in control of infection rather than full resolution, a status sometimes defined as 'latency', since during this phase there are unlikely to be any clinical indicators of disease. The combination of cytokines produced is a key, yet complex, determinant of the host resistance to progression, as measured by the nature of clinical pathology exhibited by the host (Rook 2007). In particular, IFN has been recognised as an important cytokine in tuberculosis (Collins & Kaufmann 2001), although it does not appear to directly correlate with protection from infection progression, in either mice (Elias *et al.* 2005), cattle (Buddle *et al.* 2005), or humans (Doherty *et al.* 2002, Rook *et al.* 2005, Fletcher 2007). Protection against the establishment and/or progression of infection with tuberculous mycobacteria is almost certainly more complex than currently understood, potentially involving other T cell populations such as CD8+ T cells and γδT cells (Kaufmann & Parida 2008), mucosal antibody responses and possibly even serum antibody responses (Glatman-Freedman 2006, Hoft 2008).

1.4 *M. bovis* infection in badgers

The suspicion that badgers were involved in the epidemiology of bovine tuberculosis in domestic cattle in the UK, and their potential role as a wildlife reservoir of infection (Muirhead *et al.* 1974) prompted studies of the pathology and immune responses in both experimentally and naturally infected badgers. Badgers mount both a cell-mediated response (Southey *et al.* 2002, Dalley *et al.* 1999) and a humoral response (Mahmood *et al.* 1987a) to *M. bovis* infection. However, the magnitude of the cell mediated response may be restricted in comparison with other species (Little *et al.* 1982, Morris *et al.* 1978). Badgers are poorly responsive to the intra-dermal skin test, the current routine diagnostic test for cattle, which relies on a cell-mediated response to the injection of tuberculin, and

develop less severe cellular granulomatous lesions in affected tissues such as lung and lymph nodes (Gallagher *et al.* 1976, Gavier-Widen *et al.* 2001).

In some early experimental work in badgers an initial cell-mediated response was observed following infection with *M. bovis*, with no detectable humoral element, followed by fluctuation in the cell-mediated component, and a final humoral response alone, associated with the end-stages of infection (Mahmood *et al.* 1987b). This is consistent with the hypothesis that a failing cell-mediated response with a reciprocal rise in the humoral response, is associated with the progression of infection, and is supported by work on *M. bovis* infection in cattle (Ritacco *et al.* 1991, Welsh *et al.* 2005). However, as discussed above, it is clear that protection from progressive infection in all species including the badger is a complex interaction between the host and the pathogen. Advances in immunological techniques are likely to improve our understanding of the relative importance of particular aspects of the immune response to *M. bovis* infection in badgers.

In common with other species and other mycobacterial infections, *post-mortem* examination suggests that inhalation is the main route of infection in badgers (Jenkins *et al.* 2008b). The size and distribution of lesions in lung tissue is likely to be associated with both the duration of infection and the effectiveness of any immune response mounted by the badger. *Post-mortem* examination has revealed a spectrum of lung pathology, from small numbers of discrete lesions (circa 1-2mm diameter), to larger foci of coalescing lesions, through to wide dissemination, causing almost complete consolidation of lung tissue (Gallagher *et al.* 1976). Infection may be controlled within the pulmonary system and there is evidence to suggest that in some cases infection may resolve (Gallagher *et al.* 1998). Failure to control or resolve infection results in mycobacterial multiplication within the pulmonary system and/or haematogenous dissemination, with the kidney as the main predilection site (Gallagher *et al.* 1976, Little *et al.* 1982). It has been suggested that breakdowns in host immune response, with associated progression of infection, may be associated with physiological stressors such as competition for food, territorial defence and reproduction (Gallagher & Clifton-Hadley 2000). However, the correlation of these events with detectable immune responses and pathology is poorly understood.

Interestingly, more aggressive pathology has been observed in association with positive culture from bite wounds, suggesting that infection acquired through bite wounds may result in more rapid fulminant disease (Gallagher & Nelson 1979). This hypothesis is further supported by experimental work in which badgers infected by intra-dermal injection exhibited progressive systemic infection (Pritchard *et al.* 1987) and data from the RBCT

which suggested that infection arising from bite wound transmission is more likely to be associated with widespread severe disease (Jenkins *et al.* 2008b). An alternative hypothesis is that disseminated disease originating from respiratory infection leads to preferential bacillus location and multiplication at active bite wound sites (Clifton-Hadley *et al.* 1993).

1.4.1 Diagnosis of *M. bovis* infection in badgers

Diagnosis of bovine tuberculosis infection in the live badger has, to date, been based on detection of specific immune responses and/or culture of *M. bovis* from sputum, faeces, urine, and swabs from bite wounds or abscesses. Diagnostic methods using microbiological culture have a very high specificity, and positive culture from tissues sampled *post-mortem* using a standard protocol (Pritchard *et al.* 1986, Clifton-Hadley *et al.* 1993) is deemed the gold standard against which other diagnostic tests are validated. The sensitivity of microbiological culture of samples taken from a live badger is however poor, and has been estimated at only 20% (Pritchard *et al.* 1986). This is likely to be due, in part, to the intermittent excretion of bacilli in infected badgers (Little *et al.* 1982, Clifton-Hadley *et al.* 1993, Gallagher & Clifton-Hadley 2000). Furthermore, a relatively poor sensitivity of 55% for the 'gold-standard' *post-mortem* detection method was observed when compared with a more detailed protocol (Crawshaw *et al.* 2008).

Intradermal testing for a delayed type hypersensitivity (DTH) response has been shown to be of little value in badgers in contrast to humans and cattle (Little *et al.* 1982). This is consistent with the relatively poor cellular immune response in badgers (Gallagher *et al.* 1976). Hence the development of diagnostic tests for badgers in the past concentrated on the humoral response. It is possible to detect antibody responses to an immunodominant *M. bovis* antigen MPB83, using the indirect Brock ELISA (Enzyme-linked Immunosorbent Assay) test (Goodger *et al.* 1994), and more recently in combination with other antigens in a rapid lateral flow immunoassay using the Brock TB StatPak test (Chambers *et al.* 2008). The indirect Brock ELISA test uses native MPB83 antigen and has been employed for research purposes since the mid 1990s when it was first developed. The test has a low sensitivity (range 37-54%), but relatively high specificity (range 89-98%) (Goodger *et al.* 1994, Clifton-Hadley *et al.* 1995, Greenwald *et al.* 2003, Sawyer *et al.* 2007, Chambers *et al.* 2009). The more recently developed Brock TB StatPak has a reported sensitivity of 49% and a specificity of 93% (Chambers *et al.* 2008). The sensitivity of both serological tests improves with increased severity of infection (Clifton-Hadley *et al.* 1995, Chambers *et al.* 2008).

Tests to detect cell-mediated responses were developed alongside the original serological assays (Mahmood *et al.* 1987b, Dalley *et al.* 1999, Southey *et al.* 2002), but they were time-consuming and only really suitable for research purposes. Following developments in human and cattle diagnostics, interferon gamma release assays (IGRAs) are now the focus for measures of the cell-mediated response to *M. bovis*. Initial evaluation of an ELISA test to detect IFN responses following antigenic stimulation has reported a superior sensitivity to the serological tests, at about 81% and a comparable specificity of approximately 94% (Dalley *et al.* 2008). In line with developments in the human field (Lalvani & Pareek 2009), other assays to measure IFN are being developed, including ELIspot assays measuring the number of cells producing IFN, as opposed to the amount of IFN produced, as measured in the whole blood ELISA assays (VLA 2005).

In further test evaluation studies, performance of both the serological tests and the IFN assay were found to be affected by badger age (Chambers *et al.* 2009). The sensitivity of the IFN assay was lower in cubs when compared to adults, in contrast to both serological assays which were unaffected by badger age. Test specificity was higher in cubs for all three tests, but significantly so for the Brock ELISA alone.

1.4.2 Epidemiology of *M. bovis* infection in badger populations

The maintenance and transmission of *M. bovis* within badger populations has been well studied, in particular in a free-living high-density population at Woodchester Park in south-west England, which has been intensively monitored since 1981. During that time, the spatial distribution of disease has been largely restricted (Cheeseman *et al.* 1988, Delahay *et al.* 2000a), although more recently there appears to have been an increase in the incidence of infection (Vicente *et al.* 2007a). Factors that have been associated with the persistence of infection in the population include pseudo-vertical transmission from dam to offspring (Cheeseman *et al.* 1988, Delahay *et al.* 2000a), and long survival times of potentially infected individuals (Cheeseman *et al.* 1988, Clifton-Hadley *et al.* 1993). Significant factors that drive transmission dynamics in this relatively stable high-density population include the degree and nature of individual movements between social groups (Rogers *et al.* 1998, Vicente *et al.* 2007a) and social group instability (Vicente *et al.* 2007a). These findings are consistent with culling induced perturbation of badger social structure, which has been associated with increases in infection in badgers and cattle herds (Woodroffe *et al.* 2006, Carter *et al.* 2007).

In recent years there has been an increasing recognition of the importance of social structure and individual behaviour in understanding the epidemiology of infectious disease

(Keeling 1999, Read *et al.* 2008, Cross *et al.* 2009). Both contact frequency and duration underpin disease transmission dynamics in wildlife populations, and it is possible to measure both of these parameters by collaring individual badgers with proximity loggers (Böhm *et al.* 2009, FERA 2011). Social network analysis using data collected from either observational studies or proximity loggers has been used to study bovine tuberculosis transmission amongst meerkats in southern Africa (*Suricata suricatta*) (Drewe 2010) and most recently amongst badgers at Woodchester Park in Gloucestershire (FERA 2011).

1.4.3 Correlates of infection with *M. bovis* in badgers

Understanding factors correlated with the establishment and progression of *M. bovis* infection in individual badgers may assist in explaining observed epidemiological patterns, enhance development and implementation of control strategies, and inform objective judgements about any potential negative welfare effects associated with *M. bovis* infection in badgers. Also, factors affecting physiological homeostasis have the potential to influence the effectiveness of an individual's immune response to infection.

1.4.3.1 Body weight in association with M. bovis infection

Body weight has been recorded in observational studies of badgers naturally infected with *M. bovis*, that were subsequently taken into captivity and monitored monthly for up to four years (Little *et al.* 1982). The results showed little or no observed change in weight other than the anticipated seasonal patterns. Only when the animals were close to death due to advanced tuberculosis, was there detectable weight loss. *Post-mortem* examinations have also confirmed weight loss and emaciation in the terminal stages of tuberculosis (Clifton-Hadley *et al.* 1993). In contrast, badgers which were killed for disease control purposes and subsequently found to be *M. bovis* positive on the culture of tissue samples, were generally found to be in good body condition (Clifton-Hadley *et al.* 1993). Consistent with clinical effects occurring late on in infection progression is the observation that mortality rates of infected badgers were only significantly greater than those of uninfected badgers, once *M. bovis* had been detected at live sampling (Wilkinson *et al.* 2000).

Hence, the available evidence is consistent with deterioration in body condition becoming apparent only during the later stages of disease. However, it is difficult to separate cause and effect, and an alternative explanation for these observations is that weight loss due to other factors may lead to immune compromise, precipitating the onset of progressive infection followed by death. Indeed in humans infected with *M. tuberculosis*,

a bi-directional association of body condition and tuberculosis appears to be well-accepted (Macallan 1999).

1.4.3.2 Badger sex and reproduction in association with M. bovis infection

Sex hormones influence the immune system, and as a general rule, androgenic hormones (for example testosterone) suppress immune responses, while oestrogenic hormones (for example oestrogen) stimulate immune responses (Shuurs & Verheul 1990). Males may therefore be expected to be more frequently and more heavily parasitised than females (Schalk & Forbes 1997). Published data on the prevalence of *M. bovis* infection in wildlife species are equivocal. There was no reported sex related difference in the likelihood of infection in *M. bovis* infection in wild African buffalo (*Syncerus caffer*) (Rodwell *et al.* 2001), farmed cervids (Munroe *et al.* 2000), and captive white-tailed deer (*Odocoileus virginianus*) (Palmer *et al.* 2000). However, a higher prevalence was reported in wild female Kafue lechwe (*Kobus leche kafuensis*) in Zambia (Gallagher *et al.* 1972).

Examining sex differences in point-prevalence of infection alone, however, is an oversimplification, since it is a measure that combines the likelihood of exposure to infection, and the effects of the infection on individual survival. Pathogen type (helminth, virus, bacteria, protozoa or fungus), the type of immune response, age, physiological condition, and the reproductive phase of the host (Zuk 1990, Bouman *et al.* 2005) are all important determinants of the outcome of exposure to infection. Furthermore, it may be difficult to assign any observed differences to hormonal effects alone, since there may be confounding influences of sex for example on behaviour, which in themselves may affect both the likelihood of exposure to the pathogen and the establishment and/or progression of infection.

With respect to *M. bovis* in badgers, most of the evidence suggests that males are less resilient to the progression of infection, once acquired, than females. The estimated mortality rates for badgers showing evidence of excretion of *M. bovis* were higher for male badgers than female badgers (Wilkinson *et al.* 2000), and in the same study the time from single positive culture to continued excretion was shorter in males than females. In addition, female badgers have been reported as lactating despite being detected as excreting *M. bovis* bacilli (Cheeseman *et al.* 1988, Clifton-Hadley *et al.* 1993), consistent with the hypothesis that female badgers can still reproduce and raise cubs in the face of infection. However, there are currently insufficient published reports available to determine whether *M. bovis* infection causes a decrease in female reproductive performance.

Some of these apparent sex differences may be attributable in part to infection acquired through bite wounding, as a more severe and more widely disseminated clinical picture has been associated with this route of infection (Gallagher & Nelson 1979, Clifton-Hadley *et al.* 1993, Jenkins *et al.* 2008b), and a higher incidence of bite wounding has been reported in male badgers than female badgers (Delahay *et al.* 2006a). In addition, survival times for badgers where the first culture of *M. bovis* from an individual was from a bite wound were found to be shorter than those for badgers yielding their first positive culture result from another source such as faeces, urine or sputum (Clifton-Hadley *et al.* 1993). It is, however, possible that there are genuine sex and/or endocrine differences that may be contributing to these apparent differences in mortality and progression of infection.

1.4.3.3 Clinical pathology in association with M. bovis infection

There has been limited work on the diagnostic or prognostic value of several clinical pathology parameters associated with *M. bovis* infection in badgers. Mahmood *et al.* (1988) investigated haematological parameters in captive wild caught badgers experimentally infected with *M. bovis*. An initial rise in red and white blood cell counts was followed by a fall in total haemoglobin, with a decrease in lymphocytes, but an increase in the proportion of polymorphs and monocytes late in the disease. The changes reported late in disease are not inconsistent with those seen in humans, lending some support to the use of the human pathogenesis model in the present study. However, the changes reported by Mahmood *et al.* (1988) were neither specific for tuberculosis nor statistically significant, and are therefore unlikely to be useful diagnostic tools. Work on American bison (*Bison bison*) has similarly reported raised monocytes and lymphocytes in association with *M. bovis* infection (Miller *et al.* 1989).

Chambers *et al.* (2000) examined both haematological and biochemical markers in blood samples from 16 wild badgers. Infection status was determined by *post-mortem* examination and microbiological culture. Despite the small sample size, significant elevations in copper, creatinine, gamma glutamyl transferase, GGT, and red blood cell counts were detected in infected badgers, with significant reductions in calcium and bilirubin. In isolation, however, these changes are unlikely to be specific for tuberculosis. In contrast, *elevated* calcium levels have been documented in humans with tuberculosis (Chan *et al.* 1994). Calcium levels are however associated with many other factors such as dietary intake of vitamin D and calcium, sunlight exposure, and renal function, and the prevalence of hypercalcaemia in humans with tuberculosis is itself widely variable (Chan *et al.* 1994).

In domesticated animals, acute phase proteins (APPs) are the subject of interest with respect to their value in early detection of several diseases (Cerón *et al.* 2005). The acute phase response is a non-specific reaction to tissue injury with acute phase proteins being a component of this response. Positive APPs, such as C reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA) and α_1-acid glycoprotein (AGP) increase in concentration; negative APPs, such as albumin or transferrin decrease in concentration (Cerón *et al.* 2005). Infectious diseases where APPs may have diagnostic applications in domesticated animals include feline infectious peritonitis, babesiosis, *Bordetella bronchiseptica*, ehrlichiosis, leishmaniosis, leptospirosis, parvovirus, trypanosomiasis, equine influenza and equine herpes virus (Eckersall 2006). The diagnostic value of acute phase proteins has also received some attention in human tuberculosis (Choi *et al.* 2007, Siawaya *et al.* 2008), but due to the their lack of specificty, their clinical value in isolation may be limited (Breen *et al.* 2008).

Other biomarker research, predominantly relating to humans and *M. tuberculosis* infection, includes the elucidation of host gene expression profiles which have been found to differentiate between different infection states (Mistry *et al.* 2007); proteomic profiling, which involves analysing serum for a whole spectrum of proteins, producing a "fingerprint" specific for *M. tuberculosis* infection (Agranoff *et al.* 2006); and metabolomic profiling which involves analysis of samples for metabolites associated with mycobacterial growth and multiplication (De Carvalho *et al.* 2010).

1.4.3.4 Co-infections in association with M. bovis infection

A further mechanism underlying the complexity of the pathogenesis of tuberculous infections in mammals is the effect of co-infections. Studying the effects of co-infection is inherently highly complex and must take into account the sequence of infection acquisition, biology of the pathogens, the host resources utilised and the host immune response stimulated, whilst controlling for other confounding factors such as sex, age and season (Telfer *et al.* 2008). This type of research lends itself to a probabilistic approach.

In humans, the synergistic effect of HIV and tuberculosis co-infection is having a major impact on human health, by magnifying the burden of disease, particularly in Africa (Kwan & Ernst 2011). In addition, the role of helminth infections potentially compromising the host's ability to control mycobacterial replication has been the subject of work in humans (Bentwich *et al.* 1999, Elias *et al.* 2001, Resende Co *et al.* 2006). In cattle, IFN levels specific to *M. bovis* were found to be reduced, and the severity of the tuberculous infection greater in individuals experimentally co-infected with *M. bovis* and *Fasciola hepatica* (Flynn *et al.* 2009). In addition, a study of wild African buffalo (*Syncerus caffer*) with endemic *M. bovis*

(Jolles *et al.* 2008) concluded that a combination of immunological trade-offs (where the successful elimination of one pathogen was at the expense of infection with another), and increased mortality in co-infected hosts, was the likely cause of a negative correlation between *M. bovis* and helminth infections.

The role of co-infections in bovine tuberculosis in wildlife generally and in badgers in particular, has to date received little attention. Given the research in humans and the complexity of the immunopathogenesis of tuberculous infections, it is clearly worthy of further investigation, although insufficient data may preclude any meaningful findings. Wild badgers are almost certainly infected with a range of pathogens and their interaction may be an important determinant of the outcome following infection with *M. bovis*. Other pathogenic infections reported in badgers in the UK include adiaspiromycosis (Gallagher *et al.* 1998), toxoplasmosis (Anwar *et al.* 2006), intestinal coccidiosis (Anwar *et al.* 2000), helminths (Jones *et al.* 1980, Dale 2005), piroplasmosis (Pierce & Neal 1974), trypanosomiasis (Pierce & Neal 1973), herpes virus (King *et al.* 2004) salmonellosis (Dockerty 2007) and leptospirosis (Salt & Little 1977).

1.4.4 Summary

Current understanding of *M. bovis* infection in badgers suggests that the early stages of infection are not associated with any measurable negative effects on badger reproduction, body weight or survival. Where infection is advanced, then there is evidence for a period of weight loss and emaciation, with shorter survival times where bite wounding has been identified as the likely route of infection. The badger appears to be an ideal maintenance host for *M. bovis*, and pseudo-vertical transmission has been postulated as a central mechanism in this regard. In addition, there is a considerable body of evidence highlighting the significance of social structure in the epidemiology of infection for both intra-specific and inter-specific transmission.

1.5 Objectives of this thesis

The aims of the present study are to:

- Investigate associations between *M. bovis* infection and physiological homeostasis in badgers by answering the following:
    - How is variation in badger body condition related to the different stages of *M. bovis* infection, and how are relationships affected by sex and season?
    - How is variation in reproductive success related to *M. bovis* infection in female badgers?
    - How is variation in survival times, following the detection of *M. bovis* excretion, related to badger and sex and the clinical presentation of infection?
    - How are the acquisition and progression of *M. bovis* infection in cubs related to the sex, reproductive status and infection status of adults in their social group of origin?
- Utilise the quantitative results from the IFN assay to answer the following:
    - What is the predictive value of the magnitude of the IFN response with respect to infection progression in individual badgers?
    - What is the pattern of temporal progression of cell-mediated responses to *M. bovis* infection in individual badgers, and is it related to the subsequent progression of infection?
- Investigate whether variations in haematological and blood biochemical parameters are associated with *M. bovis* infection, in order to assess the following:
    - The physiological impact of infection in an individual
    - The potential value of these parameters in either the diagnosis or staging of *M. bovis* infection.
- Investigate whether variations in co-infection with intestinal endo-parasites are related to the magnitude of the cell-mediated response to *M. bovis* infection in individual badgers.

CHAPTER TWO: General Methods

2.1 Study area

Data on badger life-histories, including *M. bovis* infection status were obtained from the Food and Environment Research Agency (FERA) live trapping and sampling programme at Woodchester Park in Gloucestershire, south-west England. Research at the site has been ongoing since 1976.

The 11km^2 study area is situated on the Cotswold limestone escarpment. Altitude varies between 47m and 210m above sea level. The climate is mild and wet and the land is hilly and wooded, comprising mixed woodland, pasture and arable. Farming is mainly beef and dairy cattle, with some sheep. The predominance of agricultural grassland creates an earthworm rich habitat, which is ideal for badgers. The study site supports 21-24 contiguous social groups from which capture and sampling data were derived for the present study.

2.2 Trapping regime

Badger traps were deployed at all active main setts four times a year (two to four times a year up to 1984). A closed season of February to April inclusive was observed to avoid the capture of heavily lactating females or highly dependent cubs during this period. Traps were pre-baited with peanuts for about four days, before being set to catch for two consecutive nights, and a sufficient number of traps were deployed at each sett to avoid trap saturation. Badgers that were trapped on the first night were held in the sampling facility overnight following general anaesthesia and sampling, to avoid recapture. Traps were checked early in the morning, and additionally from December through to January, late at night, in order to release any adult females with evidence of pregnancy or lactation. Trapped badgers were transferred to individual holding cages, which were labelled with the sett of capture, and transported to the holding facility where they were placed on a metal rack, arranged in social groups to minimise the potential for infection transfer between groups. After a period of about 45 minutes to recover from trapping and transport, badgers were anaesthetised with a combination of ketamine (8mg/kg body weight; Vetalar V, Pfizer, Sandwich, UK), butorphanol (0.8mg/kg body weight; Torbugesic 1%, Pfizer, Sandwich, UK) and medetomidine (0.04mg/kg body weight; Domitor, Elanco Companion Animal Health, Basingstoke, UK) (de Leeuw *et al.*, 2004) or prior to 2001, with an intramuscular injection of

ketamine alone (20mg/kg body weight). Once under anaesthesia, badgers were removed from the holding cage for examination and clinical sampling. Following the completion of general procedures and clinical sampling, badgers were returned to the holding cage from which they had been removed and placed in lateral recumbency for recovery. Once badgers had recovered from anaesthesia, they were offered oral rehydration solution (Lectade Small Animal; Elanco Companion Animal Health, Basingstoke, UK) in drinking troughs fixed within the holding cages. Badgers from the first night of capture were held overnight and released the following morning. Badgers from the second night of capture were released later the same day. Release was at the sett of capture, close to sett entrances.

2.3 General procedures

Each badger was permanently marked with a unique ventral abdominal tattoo at its first capture event. The tattoo of each badger was then recorded on the holding cage to ensure it was returned to the same cage. Its body weight in kilograms and length in centimetres were recorded. Body length was measured to the nearest centimetre, from the tip of the nose to the distal point of the last caudal vertebra, with the badger in dorsal recumbency. The reproductive status of all female badgers was assessed by visual examination and palpation of the mammary glands. An assessment as to whether a female had ever lactated was based on the appearance of the mammary glands, teats and surrounding areas. If it was possible to express milk, this was recorded as evidence of current lactation.

2.4 Clinical sampling for *M. bovis* infection

Samples of oesophageal mucus, tracheal mucus, urine, faeces and swabs from bite wounds or abscesses were collected for *M. bovis* culture. Mucus samples from the oesophagus and trachea were collected using a flexible catheter and a syringe to aspirate material. Each catheter was then inserted into sterile saline in a Universal bottle. A small amount of saline was drawn into the end of the catheter using the syringe and then flushed out. This process was repeated three times, after which the distal end of the catheter was cut off in two or three sections using sterilised dry scissors and left to fall into the saline.

Badgers were clinically inspected for bite wounds and enlarged lymph nodes or abscesses and their location recorded. If these were open and discharging they were swabbed using a plain cotton swab, the end of which was then cut off using sterile scissors into sterile saline. Abscesses that had not burst were aspirated using a needle and syringe.

Any aspirated material was added to sterile saline in a Universal bottle and the syringe flushed using the saline three times.

Urine samples were obtained by gentle external pressure applied to the bladder, and collected into sterile Universal bottles. Faecal samples were obtained by the application of an osmotic laxative enema (Micralax; UCB Pharma Limited, Slough, UK), to stimulate defaecation. Voided faeces were then collected from underneath the holding cage into a plain sterile plastic pot some time later.

Blood samples for immunological assays for *M. bovis* infection were taken from the jugular vein into both plain Vacutainer™ tubes (Becton, Dickinson UK, Oxford, UK) and heparinised Vacutainer™ tubes.

2.5 Laboratory analysis of clinical samples

Clinical samples for *M. bovis* culture were packaged and sent by courier to FERA, York where they were decontaminated with 10% oxalic acid and centrifuged. The pellet was inoculated onto modified Middlebrook 7H11 agar slopes. Samples were cultured for a minimum of 6 weeks at ca 37°C (Gavier-Widen *et al.* 2001).

Blood samples in plain tubes were submitted to the Animal Health and Veterinary Laboratories Agency, AHVLA (formerly Veterinary Laboratories Agency), at Langford, where serum was drawn off for serological assays to detect the presence of antibodies to *M. bovis* using the Brock ELISA test (Goodger *et al.* 1994), and after July 2006 the Brock TB StatPak (Chambers *et al.* 2008). For the Brock ELISA, results were recorded both as an optical density value (OD), and a binomial result. However, use of the OD values was complicated by changes in test reagents over time, resulting in different cut-off points meaning that optical densities from different time periods were not directly comparable. Results for the Stat-Pak assay were binomial only.

Heparinised blood samples were submitted to the VLA for prompt processing for IFN assay (Dalley *et al.* 2008). Binomial results were provided, in addition to the OD values following stimulation with PPD-A, PPD-B, positive and negative control antigens.

2.6 Author's role in data collection

For three years the author assisted the Woodchester Park field team with all aspects of field work including bait marking, trap deployment, pre-baiting, trapping, transport and anaesthesia. In addition, the author regularly carried out clinical sampling. In particular, for the data collected and reported in Chapter 6 of the thesis, the author selected the social groups for more intensive study, and examined all badgers at each capture. All faecal

samples were collected, processed and faecal egg counts conducted by the author. The author visited the laboratories at VLA Langford and Aston Down to observe first-hand the serological and gamma-interferon assays.

2.7 Welfare issues of captive badgers

All scientific procedures involving live animals were undertaken under Home Office Licence. Throughout all trapping and sampling procedures badger welfare was considered a priority. During trap deployment consideration was given maximising protection from inclement weather. Following trapping, all handling of live animals was undertaken in a calm quiet manner. To minimise the stress of transport, holding cages were wedged together to minimise movement and vehicles were driven with care. In the sampling facility, the light intensity was kept low and the temperature prior to anaesthesia was maintained at 16-19°C where possible. Following anaesthesia and during recovery, to prevent hypothermia, heat lamps were directed onto badgers as required (e.g. cubs and animals in particularly poor condition).

2.8 Social group delineation

For much of the work presented here it was important to be able to allocate badgers to social groups. Each year in February-March, the territories of social groups in the Woodchester Park study area were demarcated by bait marking (Delahay *et al.* 2000b). This method involved feeding bait mixed with indigestible harmless plastic beads of a different colour/shape at each main sett for ten days. A subsequent systematic survey of the study area identified latrines which were searched for the presence of plastic pellets in faeces. Shared latrines indicated territorial boundaries. Territorial boundaries were estimated by using a combination of the Minimum Convex Polygon method, which involved joining the outermost recorded locations of pellets to describe a polygon (Mohr 1947, Hayne 1949), and records of boundary runs from field observations. A bait-marking map and associated social group delineation using data from 2005 (Figure 2.1) illustrates the social structure of the Woodchester Park badger population in that year.

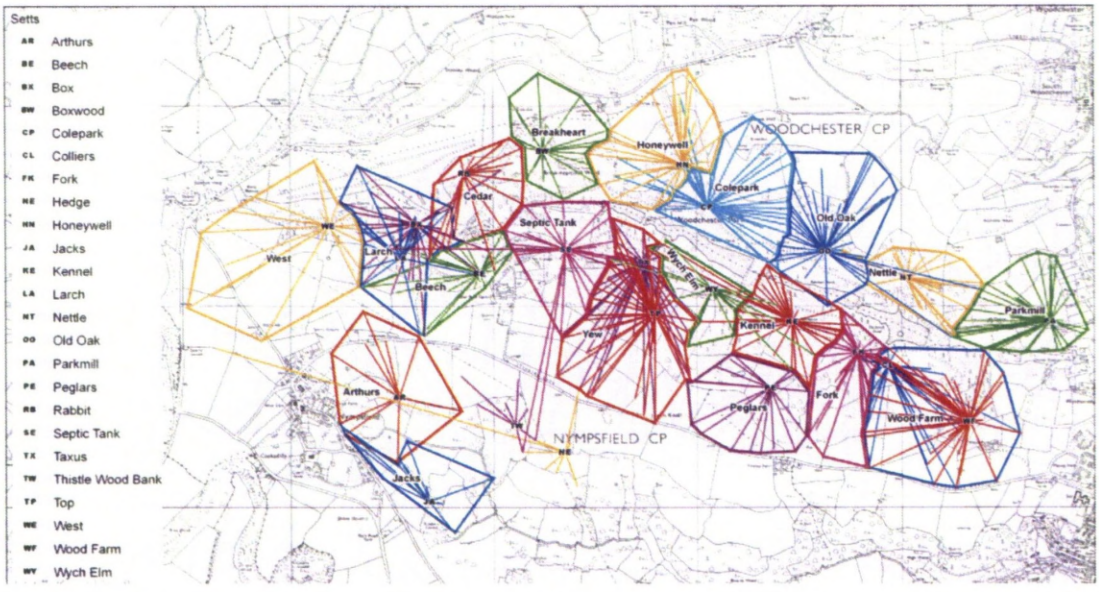

Figure 2.1. Bait marking map from the Woodchester Park study area in 2005.

The demography of the Woodchester Park badger population has been studied since 1976. An increase in the estimated population size was observed from the early 1980s through to 1999, followed by a subsequent decline and levelling off (CSL 2007). Changes in population size were related to variations in social group size, rather than in the number of social groups. These findings are consistent with the relative stability of the social structure in the face of changes in population size. However, the precise location of territory boundaries often varies amongst years, and on occasion social groups may merge or split (Fera 2011).

Mean social group sizes have been reported for various periods (see Cheeseman *et al.* 1987, Rogers *et al.* 1997) using estimates of the adult membership of each group derived from trapping data. The mean values and the range for social groups from 1978-1993 are illustrated in Figure 2.2 (after Rogers *et al.* 1997).

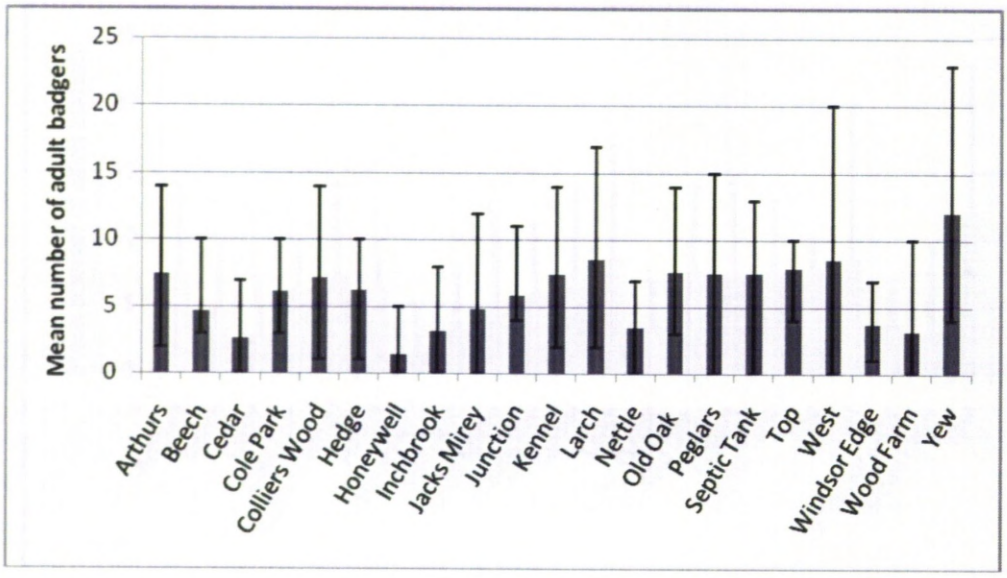

Figure 2.2. Mean number of adults per social group (1978 to 1993) estimated by Rogers *et al.* (1997). Bars indicate the range.

Movement of badgers has been well studied in the context of the epidemiology of bovine tuberculosis (Rogers *et al.* 1998, Vicente *et al.* 2007). The detection of movement amongst social groups at Woodchester Park has been largely based on trapping records, with a movement being recorded when a badger is captured in a different group to its previous capture. Some of these movements may be transient, whereas others may indicate a permanent relocation to a new social group. A study over an 18 year period from 1978 to 1995 (Rogers *et al.* 1998) revealed that just under half of badgers with capture histories had moved at some point during their observed lifetimes. Most of these were classed as occasional movers (visiting only one or two groups other than their main social group); just under 5% were frequent movers (moving between more than two social groups other than their main group); and 22% were permanent movers. Badgers of all ages were found to move, but the likelihood of movement increased with age, and most movement was to an adjacent social group.

In summary, within this high-density, undisturbed badger population the social structure appears to be spatially persistent, despite some movement between social groups, and small changes to group configuration.

2.9 Interpretation of test results

The first time a badger was detected as positive using any one of the diagnostic tests was termed the incident event for that test, but it is accepted that the true incidence of the onset of positivity for a given test remained unknown, due to the intermittent trapping and

sampling regime. However, on average, each badger was trapped twice a year in the Woodchester Park study (Delahay *et al.* 2000a), meaning that, on average, the disclosing event was likely to be no more than 6 months after the true incident event, assuming 100% test sensitivity. For analyses in which it was important to get as close to the true incident event as possible, more rigorous data restrictions were employed, and are described in detail in the relevant sections.

2.10 Assigning infection status

Using combinations of diagnostic test results, infection status was assigned to each badger at each capture event. Infection status followed a one-way classification system, potentially progressing at subsequent capture events, in a similar approach to that previously described (Delahay *et al.* 2000a, Wilkinson *et al.* 2000). Badgers moved from uninfected, to IFN positive, to seropositive, to excretor status (Table 2.1), but depending on the test results available and the hypothesis being tested, specific categories were used, details of which are given in the relevant chapters. Infection status classification was based on the hypothesis that an initial cell-mediated response is followed by a serological response as infection progresses (see Figure 5.1), a hypothesis supported by experimental infection studies in badgers (Mahmood *et al.* 1987b), and other work on *M. bovis* infection in cattle (Ritacco *et al.* 1991, Welsh *et al.* 2005).

Table 2.1. Classification system for assigning infection status to each badger at each capture event.

Infection status/diagnostic test	**IFN test result**	**Serology test result (Brock ELISA or Stat-Pak)**	**Culture of clinical samples**
Uninfected	Negative	Negative	Negative
IFN positive	Positive	Negative	Negative
Seropositive	Positive or negative	Positive	Negative
Excretor	Positive or negative	Positive or negative	Positive

2.11 Collation and analysis of data

Raw data were recorded onto an Access 2000 database (Microsoft UK) and extracted into Excel 2007 spreadsheets (Microsoft UK) for manipulation as required.

2.11.1 Potential sources of bias in the dataset

Bias in the data could arise as a consequence of capturing only a small proportion of the population and due to the movement of badgers in and out of the study area. It is probable however that a high proportion of badgers that are resident in the study area have been captured during the course of the project, as few unmarked individuals are ever found dead within or adjacent to the study area (Delahay *et al.* 2000a). Although movements, as detected by trapping records, occur frequently, permanent moves are relatively rare (Woodroffe *et al.* 1993, Rogers *et al.* 1998) so rates of emigration to neighbouring social groups outside the study area are likely to be low. In addition, few unmarked adults are captured in the study area (CSL 2008).

2.11.2 Statistical analysis and model construction

Statistical analyses were carried out using Genstat 13th Edition (VSN International, Hemel Hempstead, UK). Statistical significance was attributed to test results when $p<0.05$.

The analytical procedure used was determined by the distribution of the response variable and whether the data were based on capture events, or a summary of an individual's history. For analyses based on repeat captures of individual badgers, mixed models were utilised, incorporating individual badger as a random effect. Interaction terms between explanatory factors or variates were included where differential effects of explanatory variables on the response variable were anticipated. If the interaction terms were non-significant they were excluded, and explanatory variables included as independent factors or variates. For the mixed models, the test statistic for each of the explanatory variables was reported with its associated degrees of freedom and p value. For linear regressions with no random effects, a final model output was given, reporting the variance ratio (variance associated with the model in comparison to the residual variance), with the associated degrees of freedom, and p value. In the case of logistic regressions with no random effects, the dispersion parameter (and by extension the residual mean deviance) was fixed at 1. In these cases, the model deviance was reported in association with the degrees of freedom (df) and the p value.

CHAPTER THREE: Physiological correlates of *M. bovis* infection

3.1 Introduction

Bovine tuberculosis is a zoonotic disease that also has a significant impact on livestock health and production in the UK. In the badger, disease induced physiological effects have the potential to alter individual behaviour, and affect fecundity and survival. Understanding these effects can therefore provide valuable information relating to transmission dynamics between individuals and social groups, and the impact of disease at the population level. In addition, understanding the effects of physical condition on infection acquisition and progression in an individual is likely to improve our ability to predict the effects of extrinsic factors such as reproduction, nutritional resource limitation and climatic factors on disease dynamics.

The physiological effects of the later stages of infection with *M. bovis* in badgers appear to be similar to tuberculous mycobacterial infections in other species, and include terminal weight loss and general debility (Little *et al.* 1982, Macallan 1999, Clifton-Hadley *et al.* 1993). It seems logical to hypothesise that both disease-induced condition loss, and susceptibility to disease progression arising from condition loss may occur, as supported by research in humans and guinea-pigs infected with *M. tuberculosis* (Dai & McMurray 1998, Macallan 1999). The relative importance of these processes has, however, not been assessed in wild badgers naturally infected with *M. bovis*.

Disease-induced effects on reproductive success might be anticipated, although it has been reported that infected female badgers are able to reproduce successfully and lactate (Cheeseman *et al.* 1988, Clifton-Hadley *et al.* 1993). However, a statistical analysis of any quantitative effects of infection on reproductive success has not been undertaken, and little is known of the potential for disease induced effects on population dynamics.

Inhalation is the main route of infection in badgers (Gallagher *et al.* 1998, Jenkins *et al.* 2008b), but where *M. bovis* has been cultured from bite wounds, more aggressive pathology has been observed, suggesting that infection acquired in this way may result in more severe disease (Gallagher & Nelson 1979). Lymph node abscesses, particularly in lymph nodes of the head, are also frequently observed in naturally infected wild badgers (Murphy *et al.* 2010). The underlying pathogenesis of these lesions is somewhat uncertain, but possibilities include dissemination of pulmonary infection, reaction to a local bite

wound or injury, or acquisition of infection via the pharyngeal/upper respiratory mucosa (Murphy *et al.* 2010). Regardless of the pathogenesis, associated differences in clinical outcome have not been investigated in the badger.

The outcome and progression of disease following infection with mycobacteria vary widely both within and between species, and are fundamentally influenced by the infective dose of the pathogen, its route of infection, and the host's immune response (Collins & Kaufmann 2001, Cassidy 2006).

A host's immune response may be modified by intrinsic factors such as sex, reproductive status and nutritional status. Generally speaking the prevalence and intensity of parasitic infections are higher in males than females (Klein 2004), but this is likely to arise from a combination of exposure and susceptibility. With respect to reproductive and nutritional status, badgers demonstrate a degree of cyclicity. There are two peaks of mating activity in spring and late summer/autumn respectively, with most cubs being born in mid February (Cresswell *et al.* 1992, Neal & Cheeseman 1996). In addition, there is a well documented cycle of weight loss during the summer months followed by weight gain in the autumn (Cheeseman *et al.* 1987, Rogers *et al.* 1997).

The main objective of this study was to investigate the physiological effects on body condition, female reproductive success, and survival times associated with *M. bovis* infection using sequential capture data from a wild, naturally infected, undisturbed badger population. A specific aim was to investigate the direction of any association between body condition and infection status, and in particular, to assess how such effects may be related to badger sex, the clinical presentation of infection and nutritional and reproductive status.

3.2 Methods

3.2.1 Data collection

Data were obtained from the Food and Environment Research Agency's (FERA) live trapping and sampling programme at Woodchester Park in south-west England, where research at the site has been ongoing since 1976. Methods for data collection are given in Chapter Two. Capture events from 1982 to 2005 inclusive were used. Since 2005, there has been an apparent change in the performance of the Brock ELISA test with a marked increase in the number of positive results un-matched by a corresponding increase in the number of culture positive results. At the time of writing, there is no satisfactory explanation for this, hence data after 2005 have been excluded from all analyses, with the exception of the body condition index derivation.

3.2.2 Derivation of a body condition index

An objective body condition index (BCI) was constructed using data from badgers captured from 1997 (when body length was first recorded) to 2009, based on the relationship between body length and weight (see Appendix):

$W=aL^n$

where W = weight (g), L = body length (cm), a and n = constants (Le Cren, 1951).

This approach has been successfully employed in previous studies for the calculation of body condition scores for otters (Kruuk *et al.* 1987), and badgers (Woodroffe & Macdonald, 1995). Linear regression of natural logarithm (ln) W against ln L allowed estimation of constants separately for cubs, adult males and adult females, in order to calculate a body condition index for each capture event (BCI = observed W / aL^n). Hence where the BCI was greater than one, an individual badger weighed more than predicted from its length, and where BCI was less than one, an individual badger weighed less than predicted from its length.

The BCI was then standardised using the following formula:

BCI (std) = (BCI value – BCI mean (for age, season and sex)) / BCI SD (for age, season, sex)

Age was classified into four levels, cub (first year of life), juvenile (1-2 years old inclusive), adult (3-6 years old inclusive), and aged (> 6 years old). Juveniles were considered as a distinct category, since physiological and reproductive maturation continue during a badger's second year of life (Neal & Cheeseman 1996), and the proportion of

young females breeding in this population has been shown to be relatively small (Cheeseman *et al.* 1987, Rogers *et al.* 1997). Season was classified at three levels, spring/summer (March, April, May, June, July, August), autumn (September, October, November) and winter (December, January and February).

3.2.3 Infection status classification

Infection status for each capture event was classified according to a one-way progressive system, potentially changing at each capture event, using Brock ELISA test results and the results from the culture of clinical samples, similar to that described by Delahay *et al.* (2000a). Badgers moved from "negative" (both tests negative) to "seropositive" (Brock ELISA positive, culture negative), to "excretor" (Brock ELISA positive or negative and culture positive).

3.2.4 Statistical Analyses

3.2.4.1 Body condition and infection status

To investigate associations between body condition and infection status, a generalised linear mixed model (GLMM) was constructed, with the body condition index as the response variable. The explanatory variables were the infection status recorded at each capture event (negative, seropositive or excretor), age (cub, adult 1-6 or elderly i.e. >6), and sex, with individual badger fitted as a random effect.

3.2.4.2 Does poor body condition predispose to the onset of excretion?

The dataset was restricted to captures of badgers that were detected as excreting at some time during their life, but only included observations prior to the point at which excretion was detected. This permitted investigation of the temporal changes in body condition in the time period leading up to the detection of excretion. The dataset was further restricted to only include captures up to a maximum of 18 months prior to excretion detection for each individual badger. A period of 18 months was chosen to represent a time period during which it should be possible to detect any significant loss in condition prior to excretion detection, at the same time as being close enough to the excretion detection event to have confidence that the two events might be related. Time was described in periods of 60 days (two months). GLMMs with BCI as the response variable were run separately for males and females. Explanatory variables included the time prior to excretion

detection, badger age (cub, adult, elderly) and reproductive activity recorded at the capture event (for females), with individual badger fitted as a random effect.

3.2.4.3 Does the onset of excretion predispose to loss of body condition?

To investigate temporal changes in body condition following the detection of excretion and associations between the clinical presentation of excretion and any change in body condition, the dataset was restricted to captures of badgers subsequent to the first detection of excretion. GLMMs were constructed separately for males and females with body condition as the response variable. Explanatory variables were the time in days (described as above), badger age (cub, adult, elderly), reproductive activity recorded at the capture event (for females), and the clinical presentation of excretion, with individual badger fitted as a random effect.

The clinical presentation of excretion was categorised according to the origin of samples that were culture positive at and subsequent to the excretor incident event as described below (Table 3.1). Bite wounding has been proposed as a likely route of natural infection in badgers (Gallagher & Nelson 1979, Clifton-Hadley *et al.* 1993, Jenkins *et al.* 2008b). Lymph node abscesses are also well documented (Jenkins *et al.* 2008b, Murphy *et al.* 2010) although their pathogenesis is less clear. For the purpose of this study, it is the clinical presentation that is of interest, rather than the underlying pathogenesis. The clinical presentations observed in culture positive badgers were separated into three categories. Classification as a standard clinical presentation involved no positive cultures from bite wounds or lymph node abscesses at any capture event, but any combination of tracheal/oesophageal mucus, faeces or urine positive culture results. For a lymph node abscess clinical presentation, there had to be a positive culture from a lymph node at the excretor incident event, no culture positive bite wounds at any capture events, and any combination of tracheal/oesophageal mucus, faeces or urine positive culture results. For a bite wound classification, there was a positive culture from a bite wound at the excretor incident event (and possibly beyond), no culture positive lymph node abscesses at any capture event, and any combination of tracheal/oesophageal mucus, faeces or urine culture results (Table 3.1).

Table 3.1. Criteria for classification of the clinical presentation of excretion for badgers detected as excreting *M. bovis* by culture of clinical samples.

Category	Samples positive for *M. bovis*					
	Oesophageal/tracheal aspirate, faeces, urine		Lymph node abscess		Bite wound	
	At excretor disclosing event	Subsequent to disclosing event	At excretor disclosing event	Subsequent to disclosing event	At excretor disclosing event	Subsequent to disclosing event
Standard	YES	YES/NO	NO	NO	NO	NO
Lymph node abscess	YES/NO	YES/NO	YES	YES/NO	NO	NO
Bite wound	YES/NO	YES/NO	NO	NO	YES	YES/NO

3.2.4.4 Is there any evidence for significant effects of reproductive or nutritional stress on the associations between body condition and infection status?

GLMMs were constructed to investigate whether associations between body condition and infection status were related to season and /or reproductive status in adults of reproductive age (≥2 years old). All models were constructed with individual badger fitted as a random effect, and controlled for age (adult and elderly).

Models were run on badger capture data for the period May to August inclusive, 1982-2005 inclusive, separately for males and females. For males, this represents a period of physiological and nutritional stress associated with limited food resources (Kruuk 1978). For females, the same applies, but in addition, it may represent a period associated with the physiological consequences of lactation (Neal & Cheeseman 1996). Reproductive activity was therefore included as a factor in the analysis, such that if evidence of lactation was detected from March through to December, that female was described as reproductively active for all captures that year.

Additional models were run on badger capture data for the period September to December inclusive, separately for males and females. This is a period when weight is usually gained by both sexes due to an increase in food availability in the late

summer/autumn (Cheeseman *et al.* 1987). As above, the reproductive activity of females was included, to assess the extent to which they were able to recover body weight following lactation, and whether this varied with excretion status. For adult males this period may be associated with a second peak of reproductive activity, which in combination with excretion status may affect body weight gain following the nutritional stress of the summer (Cresswell *et al.* 1992).

3.2.4.5. Survival times following the detection of excretion

The time from detection of excretion to the last capture was not normally distributed and ranged from zero (where excretion was detected at an individual's last capture event) to 3298 days. The data were therefore grouped as follows: zero (excretion incident = last capture); medium (1-360 days); long (>360 days), resulting in a categorical response variable with three levels. An ordinal regression model was therefore constructed to investigate factors associated with the time from detection of excretion to the last capture event (response variable). The dataset of excretor badgers was further restricted, by examining data up to 2009 to confirm that badgers recorded with a last capture as far back as 2001 were in fact not captured again (resulting in the exclusion of five badgers), leaving 145 animals. Explanatory variables were the clinical presentation of excretion category (Table 3.1), age at excretion (cub, adult, elderly) and sex. A term for the interaction between clinical presentation of excretion and sex was also included in the model.

3.2.4.6 Reproductive success in female badgers excreting M. bovis

Capture data for excretor females of reproductive age (≥2 years old) were summarised to show the number of badgers that were detected as reproductively active for each 365 day period following the initial detection of *M. bovis*.

To investigate statistical associations between infection status and reproductive activity, the dataset was restricted to captures of females of reproductive age in all infection states (n=2611). A GLMM was constructed to estimate the likelihood of being detected as reproductively active, controlling for the season of capture (as reproductive activity is likely to be more difficult to detect later in the year), with individual badger fitted as a random effect. Captures from March to August inclusive were recorded as spring/summer; from September to November inclusive as autumn and December to February inclusive as winter.

To detect effects of excretor status on reproductive success, the number of cubs captured in a given year in a social group was used as a proxy for reproductive success.

Each social group/year combination for the period March to December inclusive was assigned a status based on the infection status of reproductively active female badgers captured there in that period (Table 3.2). A GLMM with a Poisson distribution was constructed with the number of cubs captured in each social group/year combination from May to December as the response variable. Explanatory variables were the social group year category as described, and the number of reproductively active females captured in that social group year combination (log transformed), with year fitted as a random effect to control for inter-annual variation.

Table 3.2. Criteria for categorising each social group/year combination (n=706 for the period 1982 to 2005), based on the excretion status of reproductively active female badgers captured during the period March to December.

Criteria	**Social group year category**
Infection status negative reproductively active females badger(s) captured from March to December	Negative
Seropositive status (excretor negative) reproductively active females badger(s) captured from March to December	Seropositive
Excretor status reproductively active female badger(s) captured from March to December, regardless of serological status	Excretor

3.3.1 Body condition and infection status

There was marked seasonal fluctuation of badger body condition during the year. Periods of reproductive and nutritional stress for females and males were superimposed on the temporal trends in body condition (Figure 3.1).

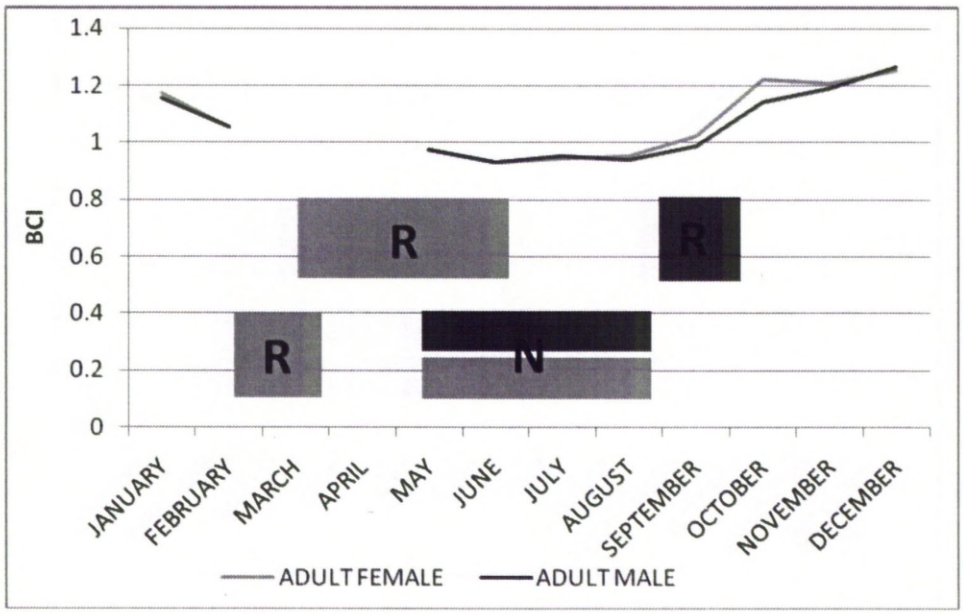

Figure 3.1. Seasonal changes in mean non-standardised BCI for adult male (dark grey) and adult female (light grey) badgers. The shaded boxes indicate approximate periods of reproductive ("R"), and nutritional ("N") stress for males (dark grey) and females (light grey).

The body condition of badgers was associated with infection status, such that those with previous or current evidence of excretion were in significantly poorer condition than those with previous or current evidence of seroconversion, and those with no evidence of previous or current *M. bovis* infection (Table 3.3 and Figure 3.2). Badger sex and age were not significant factors.

Table 3.3. Results of a GLMM investigating factors associated with variation in the standardised body condition index for badger captures (n=3790 capture events from 1982 to 2005 inclusive). Individual badger was included as a random factor in the model.

Explanatory variable	Direction of effect	Test statistic	P value
Infection status	Excretor<seropositive ≈negative	$F_{(2,2760.9)} = 5.34$	**0.005**
Sex	NA	$F_{(1,716.1)} = 0.95$	0.330
Age	NA	$F_{(1,3263.9)} = 0.36$	0.695

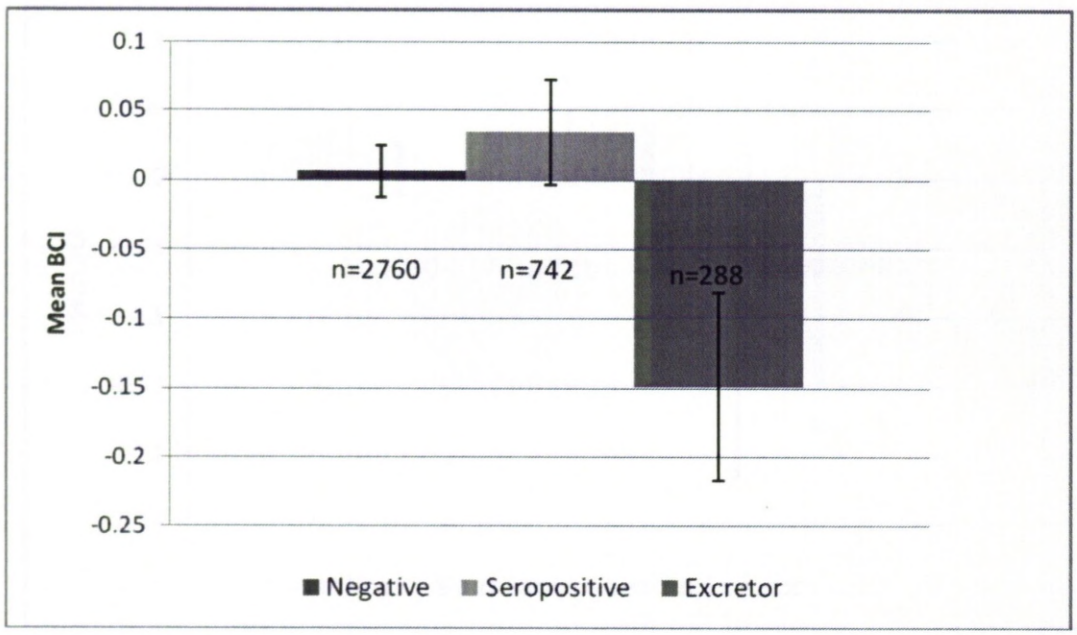

Figure 3.2. The mean standardised body condition index for TB test negative, seropositive and excretor status badgers (n=3790 from 1982 to 2005 inclusive). Error bars represent SEM.

3.3.2 Body condition prior to the detection of excretion

There was no significant association between the length of time prior to the detection of excretion and body condition for either male or female badgers (Figures 3.3, 3.4 and Table 3.4).

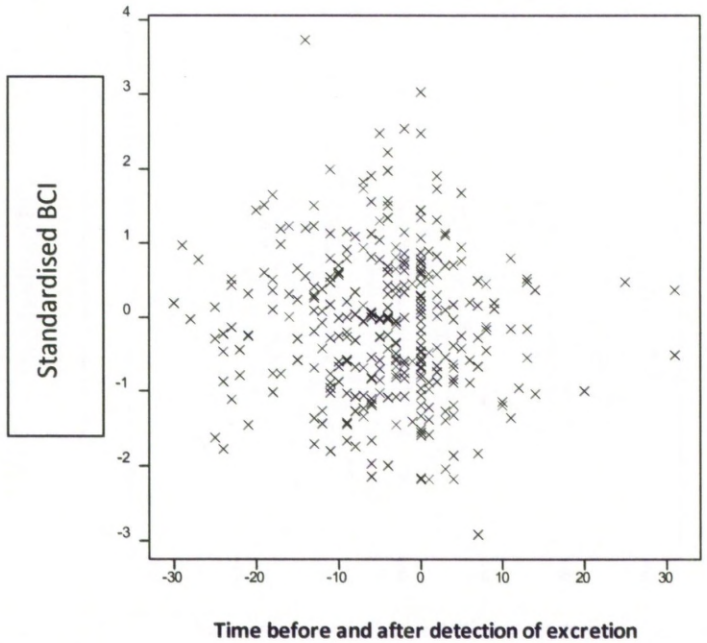

Figure 3.3. BCI against time before and after the detection of excretion for male badgers from 1982-2005 inclusive. Each unit of time represents 2 months.

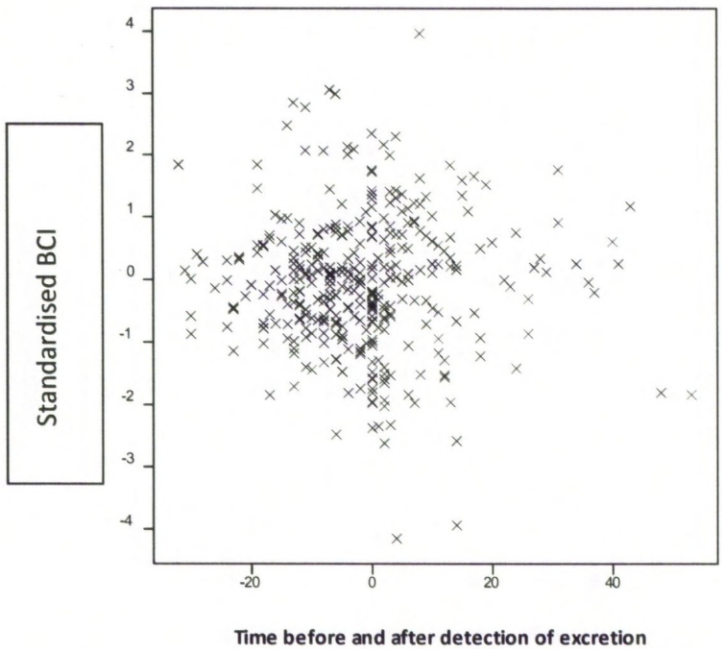

Figure 3.4. BCI against time before and after the detection of excretion for female badgers from 1982-2005 inclusive. Each unit of time represents 2 months.

Table 3.4. Results of separate GLMMs for males and females investigating variation in body condition of badgers relative to the length of time prior to the detection of excretion, reproductive activity (for females) and age for 290 captures of 163 individuals detected as excreting *M. bovis* (1982 to 2005 inclusive). Individual badger was included as a random factor in both models.

Sex	Explanatory variable	Test statistic	P value
Males (N = 157)	Time to detection of excretion	$F_{(1,149.4)} = 0.47$	0.494
	Age	$F_{(2,109.6)} = 0.07$	0.932
Females (N = 133)	Time to detection of excretion	$F_{(1,127.7)} = 1.46$	0.229
	Age	$F_{(2,76.4)} = 0.17$	0.847
	Reproductive activity	$F_{(1,48.2)} = 0.09$	0.768

3.3.3 Body condition subsequent to the detection of excretion

For male badgers only, body condition declined significantly with time following the detection of excretion (Figures 3.3 and 3.4, Table 3.5). In contrast, for both sexes there were no significant associations between body condition and the clinical presentation, age, and reproductive activity in the case of females (Table 3.5).

Table 3.5. Results of separate GLMMs for males and females, investigating variation in badger body condition relative to the time elapsed since the detection of excretion, clinical presentation and reproductive status (for females) for 262 captures of 150 individuals detected as excreting *M. bovis* (1982 to 2005 inclusive). Individual badger was included as a random factor in both models.

Sex	Explanatory variable	Direction of effect	Test statistic	P value
Males (N = 105)	Clinical presentation of excretion	NA	$F_{(2,41.7)} = 1.68$	0.199
	Time from detection of excretion	Negative	$F_{(1,98.8)} = 4.84$	**0.030**
	Age	NA	$F_{(2,92.7)} = 1.27$	0.286
Females (N = 157)	Clinical presentation of excretion	NA	$F_{(2,42.4)} = 0.47$	0.626
	Time from detection of excretion	NA	$F_{(1,139.5)} = 2.13$	0.147
	Age	NA	$F_{(2,146.8)} = 2.23$	0.111
	Reproductive activity	(Active	$F_{(1,120.5)} = 2.89$	0.091

3.3.4 Seasonal and sex effects in association with body condition and infection status

For capture events of male badgers of reproductive age (≥2 years old) from May through to August inclusive, infection status was significantly related to condition, such that excretor badgers were in poorer condition than seropositive or negative badgers (Table 3.6). For female badgers of reproductive age (≥2 years old) infection status was also a significant factor, such that excretor badgers were in poorer condition than seropositive or negative badgers. In addition, reproductive activity per se was significantly negatively associated with body condition in female badgers.

For capture events of male badgers of reproductive age from September through to December inclusive, excretion status was still significant negatively associated with body condition, although test statistics indicated a weaker association (Table 3.6). In contrast, for captures of female badgers of reproductive age, neither infection status nor prior reproductive activity that year were significantly associated with body condition (Table 3.6).

Table 3.6. Results from GLMMs for male and female badgers separately, investigating variations in body condition relative to season, age, reproductive and infection status (1982 to 2005 inclusive). Individual badger was included as a random factor in all models.

Data restriction	Explanatory variable	Direction of association	Test statistic	P value
Males ≥2 years old May-August inclusive	Infection status	Excretor>Seropositive≈Negative	$F_{(2,291.9)} = 6.93$	**0.001**
	Age	NA	$F_{(1,294.4)} = 1.04$	0.310
Females ≥2 years old May-August inclusive	Infection status	Excretor>Seropositive≈Negative	$F_{(2,351.5)} = 5.28$	**0.005**
	Age	NA	$F_{(1,525.9)} = 0.13$	0.719
	Reproductive activity detected March-December	Negative	$F_{(1,506.8)} = 32.60$	**<0.001**
Males ≥2 years old September-December inclusive	Infection status	Excretor>Seropositive≈Negative	$F_{(2,133.7)} = 3.16$	**0.045**
	Age	NA	$F_{(1,152.4)} = 0.10$	0.758
Females ≥2 years old September-December inclusive	Infection status	NA	$F_{(2,291.9)} = 0.00$	0.997
	Age	NA	$F_{(2,288.8)} = 0.84$	0.361
	Reproductive activity detected March-December	NA	$F_{(1,283.1)} = 0.09$	0.759

3.3.5 Survival times following the detection of excretion

The time from detection of excretion to last capture was significantly shorter in male badgers than in females, such that a female badger was 2.5 times more likely than a male badger to be captured after more than a year following the detection of excretion. The clinical presentation of excretion was also a significant factor in the model such that the survival time for badgers with lymph node abscesses or bite wounds at the excretion incident was significantly shorter than for badgers with a standard presentation (Table 3.7). Age at detection of excretion was non-significant. An interaction term between the clinical presentation of excretion and sex was non-significant, indicating that regardless of the

clinical presentation, sex was significant, and vice versa. It was therefore excluded from the final model.

Table 3.7. Results from an ordinal regression to investigate factors associated with the time from detection of excretion of *M. bovis* to last capture for 145 badgers (1982-2005 inclusive).

Variable	Reference level	Estimate	Odds ratio OR	95% CI for OR	P value
Age adult	Cub	0.383	1.466	0.620 – 3.470	0.384
Age elderly	Cub	0.109	1.115	0.490 – 2.537	0.795
Sex	Female	0.921	2.511	1.337 – 4.716	**0.004**
Abscess	Standard	-1.205	0.300	0.119 – 0.754	**0.011**
Bite wound	Standard	-1.167	0.311	0.143 – 0.678	**0.003**

Model deviance = 21.3, df = 5, p <0.001

3.3.6 Reproductive success in female badgers excreting *M. bovis*

The frequency distribution of excretors captured in sequential 365 day periods following the initial detection of *M. bovis* excretion, showed that some badgers were still detected as reproductively active over 5 years later (Figure 3.5).

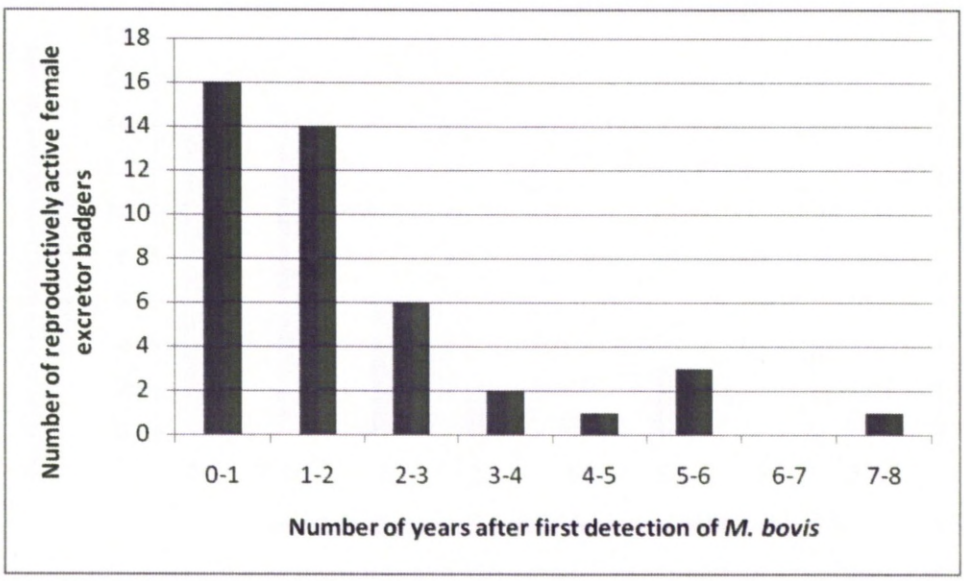

Figure 3.5. The number of badgers captured with evidence of reproductive activity relative to the time since they were first detected as excreting *M.bovis* (1982 – 2005 inclusive).

The likelihood of a badger being detected as reproductively active at a capture event was not significantly associated with infection status (negative, seropositive, excretor) in a GLMM controlling for the season of capture, age, and with individual fitted as a random effect (Table 3.8), although there was a negative trend from negative status to seropositive to excretor (Figure 3.6).

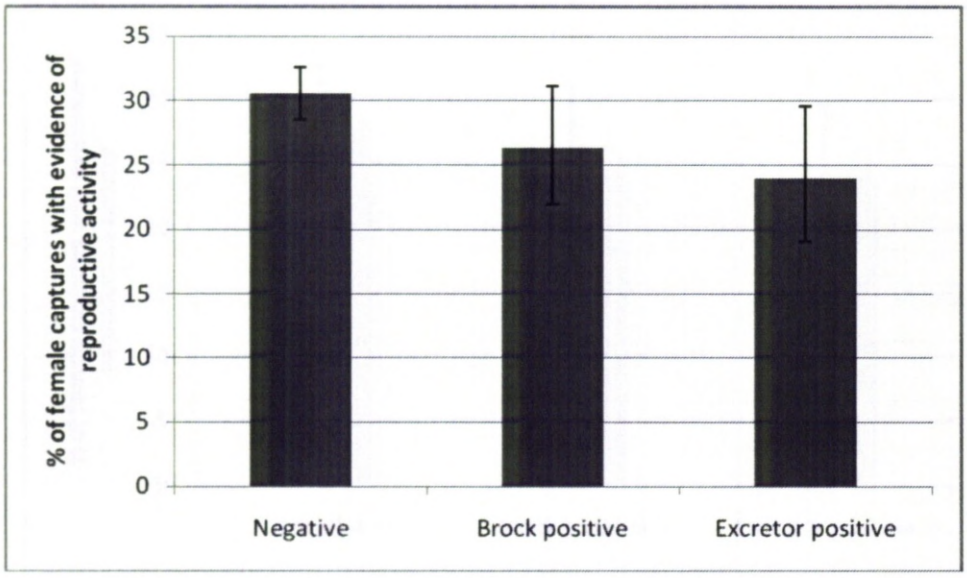

Figure 3.6. The proportion of capture events of females of reproductive age, that were detected as reproductively active relative to infection status (1982-2005 inclusive).

Table 3.8. Results from a GLMM with a binomial distribution investigating the likelihood of a female badger of reproductive age (≥2 years old) being detected as reproductively active relative to infection status and controlling for season and age (n=2611 captures from 1982 to 2005 inclusive). Individual badger was included as a random factor.

Variable	Test statistic	P value
Infection status	$\chi^2_{(2)} = 1.78$	0.168
Age (adult or elderly >6)	$\chi^2_{(1)} = 0.39$	0.535
Season	$\chi^2_{(2)} = 52.10$	**<0.001**

3.3.7 Assessing reproductive success at the social group level

Possion regression showed that the number of cubs captured in a social group from May to December was not significantly associated with the presence of test positive (as detected by either serology or culture) reproductively active female badgers within the

social group during the same period in each year (Table 3.9). These analyses controlled for the number of reproductively active females that were captured in the social group (log transformed). The mean number of cubs captured per social group, regardless of social group infection status, was 2.6.

Table 3.9. Results of a GLMM with a Poisson distribution to investigate variation in the number of badger cubs captured in a social group in a given year in relation to the presence of infected reproductively active females. Mean number of cubs captured per social group in any year was 2.6. Year was included as a random factor in the model.

Variable	**Test statistic**	**P value**
Social group infection status	$F_{(2,696.8)} = 2.26$	0.105
Number of reproductively active females captured in each social group/year (log transformed)	$F_{(1,682.8)} = 135.99$	**<0.001**

3.4 Discussion

The analyses presented here explore associations between *M. bovis* infection status and body condition for both sexes. Specific aims were to determine if a loss in body condition predisposed an individual to infection progression and/or if infection progression predisposed an individual to condition loss. Analyses also investigated how any relationship between body condition and infection status was associated with reproductive status, season and the clinical presentation of excretion (where applicable), separately for males and females. In addition, data were analysed to identify any associations between infection status and reproductive success in adult females.

3.4.1 Body condition and infection status

Badgers that were detected as excreting *M. bovis* were in significantly poorer condition than those with either evidence of seroconversion alone, or those with no evidence of infection (Table 3.3, Figure 3.2). For badgers that were detected as excreting at some point in their capture history, closer examination showed that there was no detectable significant decline in body condition prior to the detection of excretion for either sex (Table 3.4, Figures 3.3 and 3.4). However, there was a significant decline in body condition over time subsequent to the detection of excretion in male badgers only (Table 3.5, Figures 3.3 and 3.4). These findings suggest a loss of body condition in badgers with progressive disease, which is consistent with previous reports (Little *et al.* 1982, Clifton-Hadley *et al.* 1993). In addition, despite the poor sensitivity of detection of excretion as a method of detecting infection (Pritchard *et al.* 1986), the point of disclosure of excretion was correlated with a physiological change in the badger, namely detectable loss of condition, hence it may be a proxy for progressive disease in individual badgers, particularly so in males. In contrast, these results provided no evidence to support the hypothesis that loss of condition may acts as a trigger for the onset of excretion.

Evidence from the literature in humans suggests that there is a bi-directional association between body condition and tuberculosis, such that malnutrition affects the clinical presentation of tuberculosis and tuberculosis affects an individual's nutritional state (Macallan 1999). Similarly, protein malnutrition has been shown to lead to impaired immune responses to *M. tuberculosis* infection in guinea-pigs (Dai & McMurray 1998). A plausible explanation for not finding any evidence of poor condition preceding excretion in the badgers in the present study is that the seasonal nutritional stress which they experience is not sufficiently severe. Rather it is something to which badgers have become

well adapted. Indeed there is some evidence to suggest that seasonal fluctuations in body weight may be independent of food availability (Kruuk & Parish 1983). However, in years when the nutritional stress is extreme, for example during extremely hot, dry summers, when the availability of badgers' preferred food source (i.e. earthworms) may be limited, malnutrition may occur, and hence susceptibility to pathogens may increase. Future studies exploring associations between climatic factors, infection status and body condition may be of value in this regard.

Investigation of the effects of sex, reproductive status and season revealed that body condition was significantly poorer for adult male badgers that were excreting both during the summer and autumn, although there was an indication from test statistics that this difference was less significant in the autumn (Table 3.6). In contrast, body condition was significantly poorer for adult female badgers that were excreting during the summer only (Table 3.6). Furthermore, females with evidence of reproductive activity were in significantly poorer condition during the summer months only.

There is a relatively predictable and well documented temporal pattern of body condition loss through the summer followed by an increase in the autumn for adult badgers (Cheeseman *et al.* 1987, Rogers *et al.* 1997), with breeding females showing the greatest ability to increase body weight into the autumn (Woodroffe & Macdonald 1995, Cheeseman *et al.* 1987, Rogers *et al.* 1997). In addition, the nature of the badger's breeding cycle means that periods of reproductive stress are predictable. Mating behaviour has two peaks, one in spring and one in late summer/autumn (Cresswell *et al.* 1992), with the birth of most cubs in mid-February in Great Britain (Neal & Cheeseman 1996). Observational studies have shown that breeding females are in poorest condition at the end of lactation in May/June, with no loss associated with gestation (Woodroffe and Macdonald 1995). The same study showed no physiological costs for males in the spring mating period, but those that maintained testicular activity into the autumn were anaemic and had more bite wounds, suggesting physiological costs associated with the second breeding peak in the autumn. The purpose of the present study was to determine how badgers infected with *M. bovis* fared in the face of seasonal loss of body condition and reproductive stress (Figure 3.1).

Adult badgers of both sexes that were excreting *M. bovis* fared badly during the summer, when food resources were likely to be limited, and for breeding females when there were direct costs of lactation. By autumn there was no significant effect of *M. bovis* excretion on body condition in females, but excreting males were still in poorer condition.

The latter may be associated with the additional physiological and hormonal costs of a second peak in reproductive activity. In contrast, females were able to fully utilise the greater food resources available in the autumn, and gain weight to the extent that there was no significant difference in condition amongst females relative to either breeding status or excretion status. This is consistent with some research from the human literature which shows that recent childbirth does not increase mortality in women infected with *M. tuberculosis* (Burke & Sawchuk 2003), although the precise nature of the impact of pregnancy on tuberculosis in humans is a controversial subject (Snider 1984, Hamadeh & Glassroth 1992).

The findings of the present study are also consistent with a clear difference between male and female badgers in the physiological effects of *M. bovis* infection, once excretion has been detected. Females appear to show a greater ability to utilise food resources when they become available in the face of excretion and previous lactation. This is likely to also be important with respect to future breeding status as those females in the best condition in autumn are the most likely to breed in the following year (Woodroffe 1995, Delahay *et al.* 2006b).

3.4.2 Survival times following the detection of *M. bovis* excretion

Ordinal regression showed that time from detection of excretion to a badger's last capture event was significantly shorter for males than females. Also, for both sexes, the time from detection of excretion to the last capture event was significantly shorter in badgers which presented with either lymph node abscesses or bite wounds (Table 3.7). This finding is consistent with pathological and observational studies that a more severe and widely disseminated pathology is associated with infected bite wounds (Gallagher & Nelson 1979, Clifton-Hadley *et al.* 1993, Jenkins *et al.* 2008b). Although this study found evidence for more rapid disease progression and shorter survival times amongst males, there was no significant interaction between sex and the clinical presentation of excretion. This suggests that sex related differences were significant, regardless of the clinical presentation, and vice versa.

There is scant literature investigating the clinical progression of disease associated with *M. bovis* in lymph node abscesses, or the exact nature of the underlying pathogenesis. Where the head lymph nodes are affected, they may represent an alternative route of infection via the upper respiratory mucosa or may occur as an extension of pulmonary infection (Murphy *et al.* 2010). Where the abscesses do not involve lymph nodes of the head it is more likely that they are associated with undetected bite wounds, or that they

occur as an extension of pulmonary infection (L. Corner 2011, pers. comm., 13 January). The lymph node abscesses observed in the present study were largely, but not exclusively, submandibular and were not associated with bite wounds. Regardless of the pathogenesis of these lesions, it is of particular interest that badgers which were first detected as excreting *M. bovis* by isolation from lymph node abscesses deteriorated more rapidly than others. Hence such abscesses may be associated with a poor prognosis for an individual badger.

Using an individual's last capture event as a proxy for subsequent mortality assumes that badgers do not become trap averse following a trap event, and in addition, assumes that they have not moved out of the study area. In a life-table mortality analysis, Wilkinson *et al.* (2000) could find no evidence of trap-acquired shyness in this population. Permanent movement from one social group to another is also reported to be rare in high density populations (Woodroffe *et al.* 1993, Rogers *et al.* 1998), and this is supported by the low numbers of captures of unmarked adults in the study area (CSL 2008), and of marked badgers found dead outside the study area (Delahay *et al.* 2000a). The analyses of survival times from the detection of *M. bovis* excretion in the present study are again consistent with a difference between male and female badgers. Previous reports also indicate that mortality rates for badgers showing evidence of excretion of *M. bovis* were higher in males than females (Wilkinson *et al.* 2000), and that the time from a single positive culture to continued excretion was shorter in males than females (Wilkinson *et al.* 2000). It would not be unreasonable to suggest that this relationship was more to do with the clinical presentation of excretion than sex per se, with bite wounding being observed more frequently in adult males (Delahay *et al.* 2006a). However, the non-significant interaction between sex and clinical presentation of excretion in the present analysis do not support this.

Sex hormones do influence the immune system, with more vigorous humoral and cell-mediated responses observed in female mice and humans (Eidinger & Garrett 1972, Weinstein *et al.* 1984, Amadori *et al.* 1995, Giron-Gondalez *et al.* 2000). However, given the complexity of the immune response to tuberculous mycobacteria, this may be simplistic.

In humans, a long-term study in the USA found that being male was an independent risk factor for increased mortality in individuals infected with *M. tuberculosis* (Horne *et al.* 2010). The findings of the present study are consistent with a less *effective* response following the onset of *M. bovis* excretion, and higher physiological costs in male badgers,

regardless of the magnitude or balance of components of the immune response and the route of infection.

3.4.3 Reproductive success in females excreting *M. bovis*

Female badgers have been detected as lactating despite excreting *M. bovis* bacilli (Cheeseman *et al.* 1988, Clifton-Hadley *et al.* 1993). In the present study, evidence of breeding was observed in female badgers that were excreting *M. bovis* for over five years following the detection of excretion (Figure 3.5). Furthermore, the present study showed that there was no significant association between infection status and the likelihood that a female badger of reproductive age would be detected as reproductively active (Table 3.8), providing no evidence for a disease-induced reduction in reproductive activity amongst infected females. However, there was a non-significant downward trend in the likelihood of a female badger exhibiting evidence of reproduction evident with increasing infection progression (Figure 3.6), so it is possible that subtle effects do exist.

Cub production at the social group level was also investigated for infection-induced effects on reproductive success of females. This study found that the number of emergent cubs captured per social group year combination was not significantly associated with the presence of any infected reproductively active females within the social group, controlling for the number of reproductively active females captured that social group/year, and for interannual variation (Table 3.9). Since cubs are unlikely to emerge and be captured until the end of the lactation period, this finding is consistent with there being no detectable significant infection induced effects (at the levels of both seroconversion and progression to excretion) on the success of pregnancy and lactation. The analysis is limited in that there is an assumption that the cubs that were captured were produced and reared by the females detected as reproductively active in the group. This is an assumption that may be possible to address in future, utilising genetic data from hair samples that are routinely collected from all badgers at their first capture event. With respect to the epidemiological role of infected breeding females, chapters Four and Five will aim to explore the outcome for the cohorts of cubs from social groups with resident infected breeding females, in particular investigating the likelihood of acquisition of infection, body condition, and cub survival to adulthood.

Even though the analyses in the present study controlled for interannual variation, it is possible that in years of particularly poor food availability during the lactation period of March to May, (in association with very dry spring conditions for example), there could be differential effects on lactation success associated with infection status. For example, it may

be that limited food resources would negatively affect lactation (resulting in poorer early cub survival) in excretor females more than in uninfected females. Unfortunately it is probable that sample sizes would not permit a similar analysis of reproductive success in association with infection status that included climatic factors, but it is perhaps something that could be considered in future work.

Collectively, the findings of the present study support the hypothesis that there are no significant effects of *M. bovis* infection on female reproductive success. The capacity for females to continue to reproduce successfully despite infection, is further supported by the observation that females that are excreting *M. bovis* are able to regain weight following lactation, as body weight in the autumn is related to successful breeding in the following year (Woodroffe 1995, Delahay *et al.* 2006b).

In conclusion, the analyses reported here show that there are observable negative physiological effects of condition loss in badgers following the detection of excretion of *M. bovis*, but there is no evidence to support the hypothesis that poor condition associated with average seasonal and reproductive stresses precedes and potentially triggers excretion. The negative effects on body condition appear to be most marked during the summer, probably in association with nutritional stress and reproductive stress for females. Female badgers, however, appear to be more resilient to the phase of disease associated with excretion of *M. bovis* than males. Females gain body weight following the summer months to a greater extent than males, live longer than males following the detection of excretion, and continue to reproduce, with no significant effects on their reproductive success.

From an epidemiological perspective, the role of infectious female badgers may be central to the maintenance of infection in badger populations. In addition to enhanced survival times as excretors, which increases opportunities for transmission to other individuals, their ability to continue to reproduce successfully enhances the likelihood of transmission to their cubs. This may be an important factor in the observed clustering of *M. bovis* infection at the social group level in undisturbed badger populations (Delahay *et al.* 2000a).

CHAPTER FOUR: Correlates of *M. bovis* infection and disease progression in cubs

4.1 Introduction

Bovine tuberculosis is endemic in many badger populations in Great Britain and Ireland, particularly those in the south-west of England, and the badger appears to be an ideal maintenance host (Cheeseman *et al.* 1989). The importance of social structure in the epidemiology of infectious diseases is an increasingly well-recognised concept (Keeling 1999, Cross *et al.* 2004). The clearly defined social structure of high-density badger populations (Delahay *et al.* 2000a), in conjunction with the potentially long duration of infectiousness in individuals, are important factors underpinning the maintenance of *M. bovis* infection in badgers.

The annual breeding cycle of the badger ensures a relatively synchronised production of a new cohort of susceptible individuals into the population each year. The infection status of adult badgers within a social group is likely to have a significant effect on the acquisition of infection in cubs. In particular, maintenance of infection within a population will be enhanced by pseudo-vertical transmission from infectious dams to their offspring as a consequence of their close and prolonged periods of contact (Anderson & Trewhella 1985). This is supported by data from field studies of the badger population at Woodchester Park, which show that infectious females are able to reproduce successfully and transfer infection to their offspring (Cheeseman *et al.* 1988) and that the presence of infectious adult females in a social group is a significant risk factor for the incidence of infection in cubs (Delahay *et al.* 2000a). It has also been suggested that the transmission of maternal antibody could protect cubs from future disease progression (Newell *et al.* 1997), although this hypothesis was based on a descriptive analysis of data collected over a 5 year period from a single social group of badgers at Woodchester Park.

The previous chapter highlighted the potentially important role of infectious adult females in the maintenance of infection in the population, due to their prolonged survival relative to adult males, and their ability to maintain body condition and continue to reproduce successfully, despite evidence of progressive infection. It is therefore proposed that the level of risk to a cub of early *M. bovis* infection would follow a gradient, being highest in social groups containing reproductively active infected females, decreasing to

social groups with other infected adults, and decreasing again to the lowest risk in social groups in which there was no evidence of infection in the adults. Within the infected adults, a further split is proposed based on their diagnostic test status, such that groups in which *M. bovis* was detected by clinical sampling were considered likely to present a greater risk than those with evidence of seroconversion alone. The findings of Chapter Three support the detection of *M. bovis* excretion as a useful indicator of disease progression and infectiousness to other badgers.

The first aim of this chapter therefore is to expand on some of the previous findings from this population, by investigating associations between the infection status, sex and reproductive status of adult badgers resident in a social group, and the body condition, survival and the acquisition and progression of infection in cubs born into the same social group.

The second aim of this chapter is to investigate the extent of infection acquisition in cubs in the population to assist in the evaluation of disease management options. Successful implementation of a vaccination strategy, for example, depends to some extent, on the vaccination of uninfected cubs. Since cubs spend their first few weeks underground, they are unavailable to any above-ground disease management interventions during this period. Both the proportion of cubs that emerge from the sett already infected, and the proportion that are infected during their first year of life are important, since vaccination prior to acquisition of infection is considered to be a pre-requisite for protection, either from infection, or more likely to be the case with Bacille Calmette-Guérin (BCG) in badgers, protection from progression to excretion (Chambers *et al.* 2011). Estimating the likelihood of pre-emergent infection is unrealistic with the current data, but it is possible to report the likelihood that a cub is detected as infected during its first year. In addition, for those cubs that are detected as infected during their first year, analysis will assess the likelihood of that infection being acquired prior to emergence, relative to the infection status of the adults resident in the social group.

4.2 Methods

Life-history data for each individual badger at each capture event (from 1982-2005) were collected as described in Chapter Two, General Methods. For the following analyses, unless stated otherwise, the dataset was restricted to badgers that were captured as cubs and where the infection status of the social group of origin could be classified as described below.

4.2.1 Individual infection status classification

Infection status for each badger at each capture event was classified according to a one-way progressive system, potentially changing at each capture event, using Brock ELISA test results and the results from the culture of clinical samples, similar to that described by Delahay *et al.* (2000a). Badgers moved from "negative" (both tests negative) to "seropositive" (Brock ELISA positive, culture negative), to "excretor" (Brock ELISA positive or negative, and culture positive).

4.2.2 Social group infection status classification

Five categories of social group infection status were created using data from capture events for all adult badgers from each social group for the period March through to the following January inclusive for each year. Negative (N) groups were defined as those in which all adult badgers captured that year were of negative serological and excretor status. Seropositive groups (S) were those in which at least one serological status positive, excretor status negative adult badger was captured (excluding reproductively active females). Excretor groups (E) were those in which at least one excretor status positive adult badger was captured (regardless of serological status and excluding reproductively active females). Breeding seropositive groups (BS) were those in which at least one serological status positive, excretor status negative reproductively active female was captured and finally, breeding excretor groups (BE) were those in which at least one excretor status positive reproductively active female (regardless of serological status) was captured.

In each social group in each year, a cohort of cubs was born for which the social group infection status category was a proxy for the natal environment. The frequency and proportion of social group years that fell into each category was plotted to illustrate the relative numbers of cub cohorts associated with social groups of differing infection status.

4.2.3 Correlates of seropositivity and *M. bovis* excretion in cubs

Two generalised linear models (GLM) were used to investigate associations between the social group of origin for a cub and whether it was detected as firstly seropositive, and secondly, excreting *M. bovis*, whilst controlling for the probability that the likelihood of a positive test result increased with the number of test events.

For the first model, the dataset was further restricted to exclude badgers that, although they were captured as cubs, were not subjected to any serological testing in their first year (most commonly due to no blood samples being available). A GLM with a binomial distribution was constructed with the maximal serological status acquired (positive or negative) by cubs as the response variable. Explanatory variables were the natal social group infection category and cub sex, and the number of times the cub was tested for a serological response (log transformed).

Secondly, a GLM with a binomial distribution was constructed using the original unrestricted dataset, with the result of the culture of clinical samples in cubs as the response variable. Explanatory variables were the natal social group infection status, cub sex, and the total number of times the cub was clinically sampled (log transformed).

4.2.4 The timing of detection of seropositivity in cubs

The proportion of emergent cubs that were detected as infected using the Brock ELISA test during that first year was reported for the whole study period, in conjunction with the mean number of test events for all cubs. A probability tree approach as described in Forrester *et al.* (2001) was used to estimate the increase in sensitivity associated with multiple testing.

Assuming a perfect diagnostic test, cubs infected pre-emergence would test positive at their first test event, which would also have to be reasonably close in time to emergence. Data were restricted to seropositive cubs that were also tested before September, in order to balance being as close to emergence as practical, at the same time as permitting a reasonable sample size to test associations. Test dates were standardised by conversion to an equivalent number of days after January 1st. Frequency distributions of the number of days from January 1st that positive first test events occurred were calculated for the whole dataset and for cubs relative to the infection status of social groups. A frequency distribution for the number of seropositive cubs that were detected as such at their first test event relative to their natal social group infection status was calculated. To test if the likelihood of pre-emergent infection was significantly associated with the infection status of

resident adults in a social group, a GLM was constructed using the likelihood of a cub's first capture event being positive as a binomial response variable. Explanatory variables were the social group infection status and cub sex.

4.2.5 Correlates of cub survival

To investigate cub survival, cubs that were first captured in 2005 were excluded. As this was the last year in the dataset, there was no opportunity for them to be captured as adults. The proportion of cubs that survived to be captured as adults relative to the infection status of the natal social group was plotted separately for males and females. A generalised linear mixed model (GLMM) was constructed to investigate the likelihood of a cub subsequently being captured as an adult relative to its social group infection status, sex and mean body condition as a cub, whilst controlling for interannual variation, by including year of first capture as a random effect. An interaction term between sex and the social group infection status was also included as an explanatory variable in the model.

4.2.6 Maternally derived antibody in cubs

Two specific and separate analyses were used to investigate whether there was evidence for a protective effect in cubs associated with the transfer of maternally derived antibodies to *M. bovis*. The first analysis tested the association between transient serological responses in cubs and progression to excretion, and the second tested the validity of using evidence of a transient serological response, derived from periodic capture events, as a proxy for maternally derived antibody.

Using the dataset of badgers that had a serological test as cubs, a further restriction was applied to include only those badgers that were detected as seropositive at some point during their capture history. In addition, badgers were only included in the analysis if there was an opportunity for their serological response to be classified as transient or maintained. This required that there was at least one further serological test result as adults for those that were positive as cubs, or in any year following that of the seropositive detection incident for those that were first detected as positive as adults.

A serological response was recorded as transient if, for a badger with a seropositive incident event as a cub, there were no seropositive results as an adult, and for a badger with a seropositive incident event as an adult, there were no further seropositive results in any subsequent year. A serological response was recorded as maintained if, for a badger with a seropositive incident event as a cub, there was at least one seropositive result as an

adult, and for a badger with a seropositive incident event as an adult, there was at least one seropositive result in any subsequent year.

A logistic regression model was constructed to investigate whether variation in the likelihood of *M. bovis* detection at any point in the badger's life-history by culture from live sampling was related to the type of serological response (transient or maintained), badger sex and badger age at seropositive incidence (cub or adult), controlling for the total number of times a badger was tested (log transformed).

A final model was constructed to investigate whether variation in the likelihood of detection of a transient seropositive response in a cub was related to the natal social group infection status (as described below), and the age at which a seropositive response was first detected. Badger sex was also included in the model. This was to determine whether a transient response was a valid proxy for maternally derived antibody. If there were transfer of maternally derived antibody it would be most likely to be detected in cubs that were born into social groups with resident infected females based on a serological response, and/or evidence of *M. bovis* excretion. In each year, each social group was categorised as either negative (no reproductively active females with evidence of infection present) or positive (reproductively active females with evidence of infection present, based on seropositive and/or excretor status).

4.3 Results

4.3.1 Infection status of badger social groups

For the 24 year period 1982-2005 inclusive, there were 706 social group-year combinations and a total of 1815 cubs were captured. For 46% of social group-year combinations, no evidence of infection was detected in any of the adults captured (Figure 4.1). This approximated to 325 group cub cohorts, and 835 cubs over 24 years, or 35 cubs per annum, born and reared in social groups where adults had no evidence of infection. Only 9% of social group-year combinations were associated with a breeding excretor group. This approximated to 64 group cub cohorts and 163 cubs over 24 years, or 7 cubs per annum, being born into a social group with resident breeding excretor female badgers.

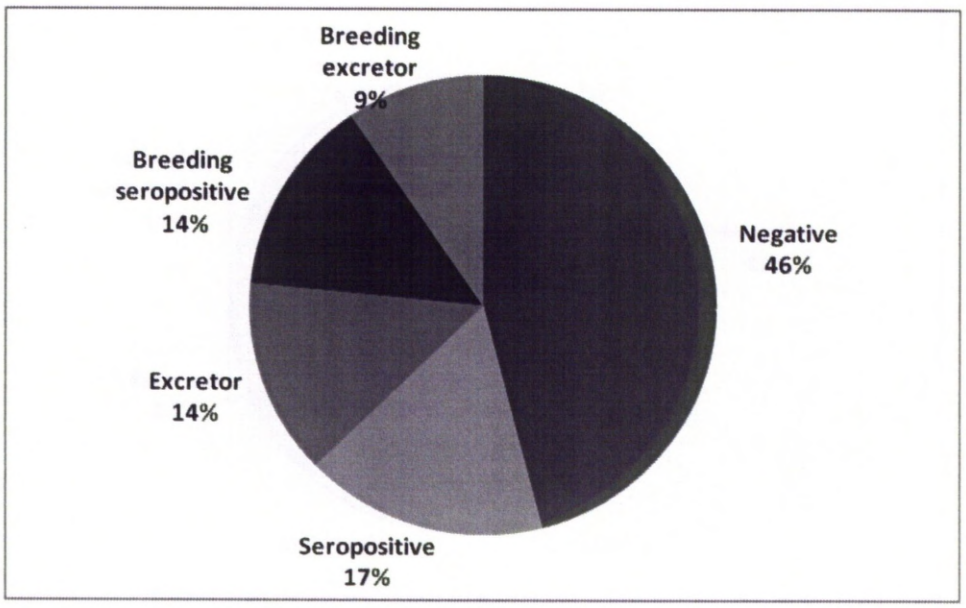

Figure 4.1. Frequency of occurrence of badger social group-years falling into different categories of infection status (1982 – 2005 inclusive).

4.3.2 Correlates of seropositivity and *M. bovis* excretion in cubs

The probability of a cub being detected as seropositive using the Brock Elisa test was significantly associated with the infection status of the social group of origin. Cubs born into excretor, breeding seropositive and breeding excretor groups were more likely to be seropositive than those born into negative groups. There was no significant difference between cubs born into seropositive groups and those born into negative groups ($p>0.05$). Furthermore, the highest likelihood of detecting a seropositive response was seen in cubs

born into social groups with a resident reproductively active excretor female badger (Table 4.1).

Table 4.1. Results from a logistic regression investigating factors associated with the likelihood of badger cubs being detected as seropositive (n=1761), using the Brock ELISA (1982-2005 inclusive).

Explanatory variables	**Estimate**	**Odds ratio**	**95% CI for odds ratio**	**P value**
Seropositive group (S)	0.275	1.316	0.653 – 2.654	0.443
Excretor group (E)	1.189	3.282	1.815 – 5.937	**<0.001**
Breeding seropositive group (BS)	1.665	5.287	3.190 – 8.762	**<0.001**
Breeding excretor group (BE)	2.132	8.436	5.038 – 14.120	**<0.001**
Number of serological tests (log transformed)	1.209	3.350	2.382 – 4.712	**<0.001**
Sex	0.008	1.008	0.728 – 1.395	0.963

Model deviance = 169, df = 6, $p<0.001$

The probability of a cub being detected as excreting *M. bovis* was also significantly associated with the infection status of the social group of origin, such that those born into breeding excretor groups were more likely to be detected as excreting, than those born into a negative group (Table 4.2).

Table 4.2. Results from a logistic regression investigating factors associated with the likelihood of badger cubs being detected as excreting *M. bovis* by clinical sampling (n=1815) (1982-2005 inclusive).

Explanatory variables	Estimate	Odds ratio	95% CI for odds ratio	P value
Seropositive group (S)	0.421	1.524	0.427 – 5.446	0.517
Excretor group (E)	0.980	2.665	0.846 – 8.395	0.094
Breeding seropositive group (BS)	1.001	4.468	0.977 – 7.581	0.055
Breeding excretor group (BE)	1.497	4.468	1.625 – 12.290	**0.004**
Number of cub captures (log transformed)	1.302	3.676	1.784 – 7.578	**<0.001**
Sex	-0.115	0.892	0.462 – 1.720	0.732

Model deviance = 28.6, df = 6, p<0.001

4.3.3 The timing of detection of seropositivity in cubs

Of the 1761 cubs captured and tested during the study period (1982-2005), 184 (10.4%; 95% confidence interval 9.1% – 12.0%) were detected as seropositive as cubs using the Brock ELISA test. The mean number of test events for all cubs was 2.3. Using a probability tree approach, and assuming independence of all tests, testing cubs twice increased the sensitivity (α) of detection of a seropositive response from a range of 37% to 53% (Goodger *et al.* 1994, Clifton-Hadley *et al.* 1995, Greenwald *et al.* 2003, Sawyer *et al.* 2007) for a single test, to a range of 60% to 78% (α+ (α(1- α)) for two tests.

Of the 184 seropositive cubs, 169 were tested before September in their first year. Of these, 42.0% (95% confidence interval 34.5% - 49.8%) were positive at their first test. The majority of these test events were during May (Figure 4.2). When broken down by social group infection status, 70.5% of the cubs born into breeding excretor groups were positive at the first test event, in contrast to less than 42% for cubs from all other groups (Figure 4.3). Furthermore, the majority of the positive first test events for cubs from breeding excretor groups were clustered in May and June, although sample sizes in some cases were small (Figure 4.4).

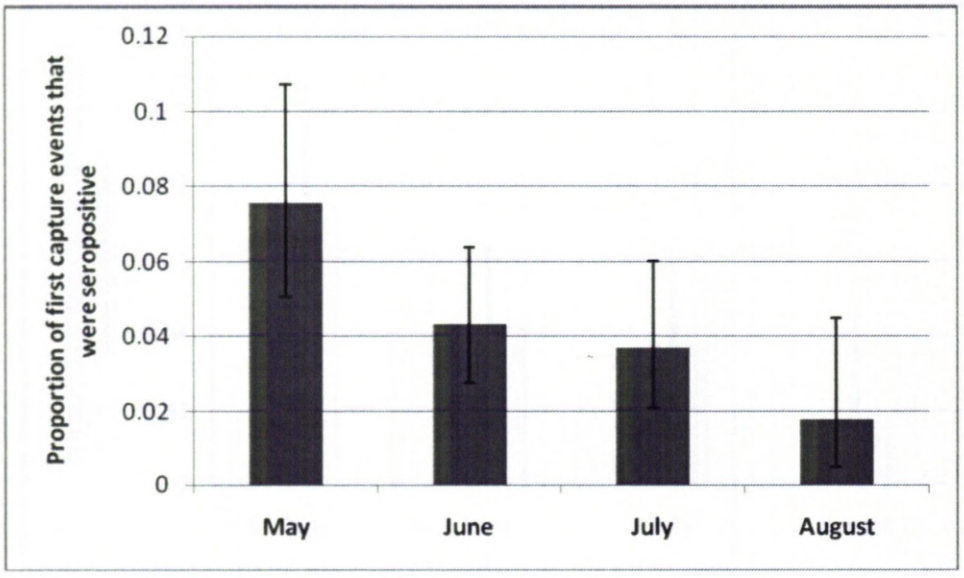

Figure 4.2. The proportion of first capture events that were seropositive for badger cubs captured before September in their first year (1982-2005 inclusive).

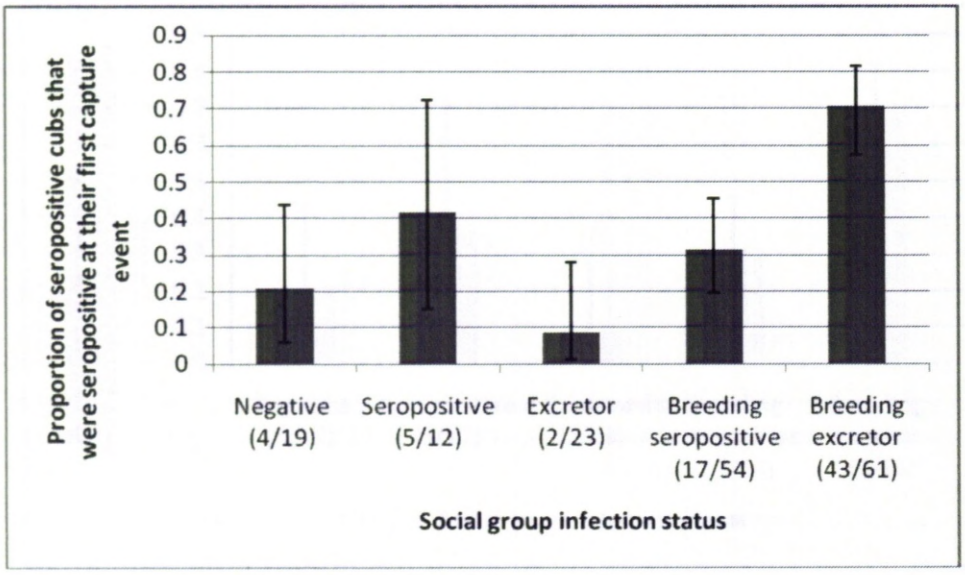

Figure 4.3. The likelihood of detection of a seropositive response at the first test event, for 169 badger cubs detected as seropositive at some point during their first year from social groups with differing adult infection status (1982 to 2005 inclusive). All 169 cubs were tested prior to September. Error bars represent 95%CI.

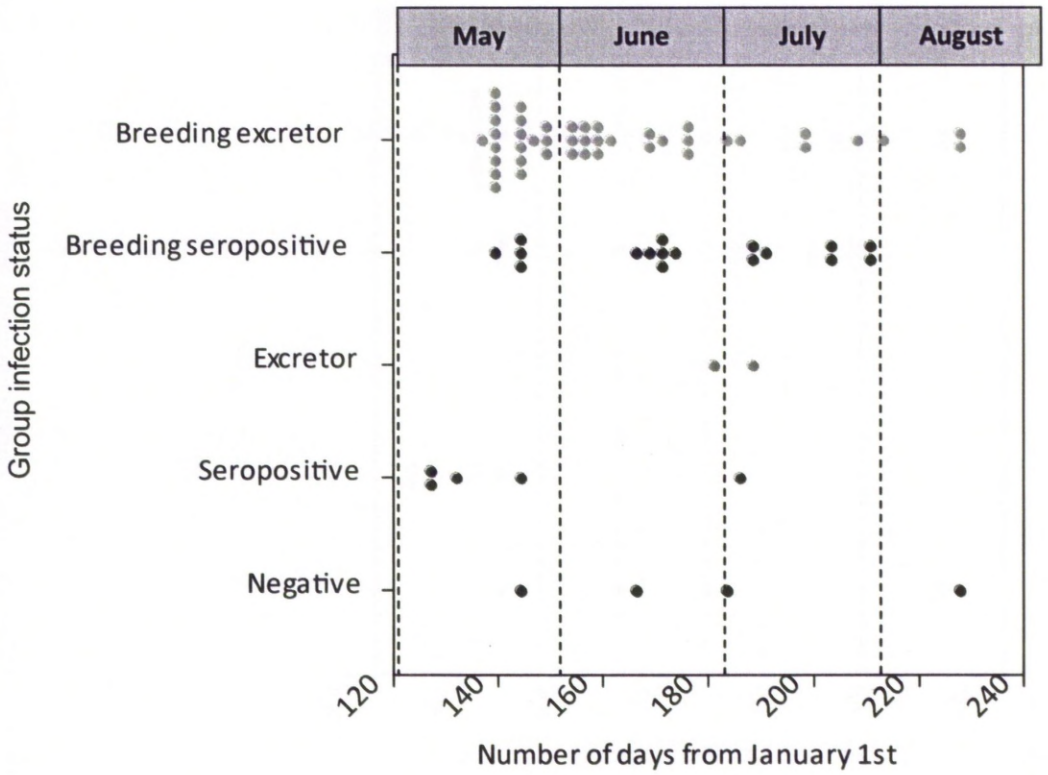

Figure 4.4. Frequency distribution of the first test events that were seropositive prior to September for badger cubs originating in social groups of differing infection status (1982-2005 inclusive).

The probability of these 169 cubs being detected as seropositive at their first capture event was significantly correlated with the infection status of the social group of origin, such that those that were born into breeding excretor groups were significantly more likely to be seropositive at their first capture event than those born into negative groups (Table 4.3). There was no significant difference between other groups of differing infection status.

Table 4.3. Results from a GLM investigating the association between the likelihood of a badger cub being detected as seropositive at its first capture event and the infection status of its social group for 169 seropositive cubs, all tested prior to September (1982-2005 inclusive).

Explanatory variables	Estimate	Odds ratio	95% CI for odds ratio	P value
Seropositive group (S)	0.969	2.636	0.536 – 12.960	0.233
Excretor group (E)	-1.061	0.346	0.056 – 2.156	0.256
Breeding seropositive group (BS)	0.523	1.687	0.485 – 5.866	0.411
Breeding excretor group (BE)	2.168	8.743	2.540 – 30.100	**<0.001**
Sex	0.127	1.136	0.567 – 2.277	0.720

Model deviance = 39.3, df = 5, $p<0.001$

4.3.4 Correlates of cub survival

For the 24 years of the study period (1982-2005 inclusive), the proportion of male cubs from breeding excretor groups that survived to adulthood was 41% in contrast to over 50% for cubs from all other groups. The likelihood of survival of female cubs ranged from just under 60% for cubs from breeding excretor groups to 67% for cubs from breeding seropositive groups (Figure 4.5). After controlling for interannual variation, the likelihood of a cub surviving into adulthood was significantly associated with social group infection status, such that cubs born into breeding excretor groups were less likely to be captured as adults than cubs born into negative groups (Table 4.4). There was no significant difference between the likelihood of survival of cubs from the other infected groups and those from negative groups ($p>0.05$). In addition, female cubs were more likely to survive than male cubs (Table 4.4). The interaction term between sex and social group infection status and the mean cub body condition were non-significant factors in the model.

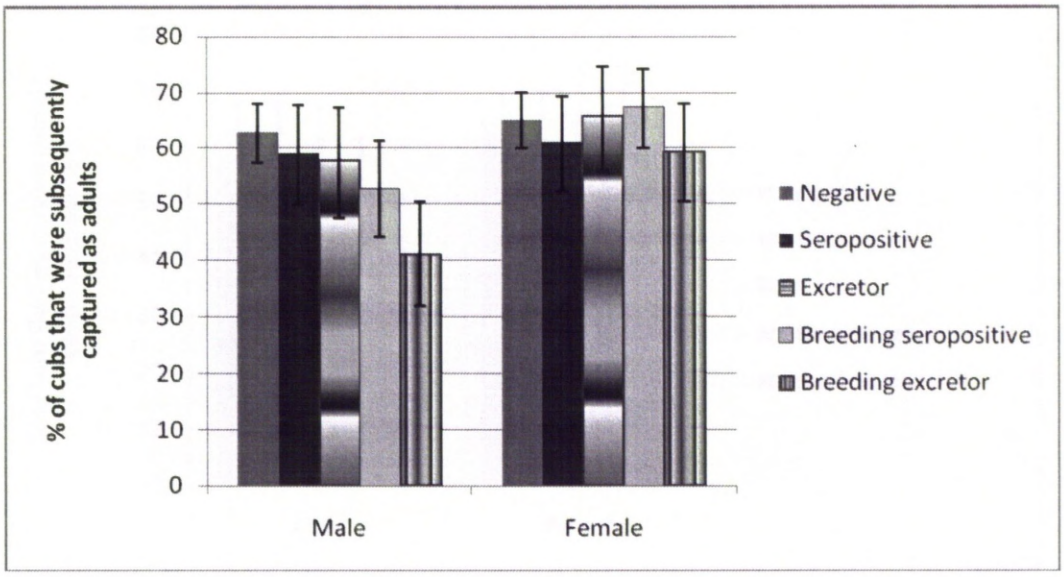

Figure 4.5.The proportion of male and female badger cubs from social groups of differing infection status, that were subsequently captured as adults (1982 – 2004 inclusive). Errror bars represent 95% CI.

Table 4.4. Results from a GLMM investigating the likelihood of badger cub survival into adulthood relative to the infection status of the social group of origin and cub sex, with year of first capture fitted as a random effect (n= 1721 badgers first captured from 1982-2004 inclusive). The non-significant cub condition score and an interaction term between cub sex and the social group of origin were excluded from the final model.

Explanatory variables	**Direction of effect**	**Test statistic**	**P value**
Social group infection status	BE <BS≈E≈SP≈N	$\chi^2_{(4)} = 2.72$	**0.028**
Sex	Male < female	$\chi^2_{(1)} = 11.95$	**<0.001**
Year of first capture – random effect	NA	NA	NA
Mean BCI as a cub	*NA*	$\chi^2_{(1)} = 0.13$	*0.719*
Interaction term SG.sex	*NA*	$\chi^2_{(4)} = 0.22$	*0.930*

4.3.5 Maternally derived antibody in cubs

Within the cohort of badgers that were detected as seropositive (n=179), the likelihood of being detected as excreting *M. bovis* at any point was significantly greater in animals with a maintained serological response compared to those with a transient response (Table 4.5). Age at detection of the serological response and sex were non-significant factors in the model.

Table 4.5. Results from a logistic regression to investigate factors associated with the likelihood of detection of *M. bovis* by culture from live sampling in 179 badgers that were detected as seropositive either as cubs or adults (1982-2005).

Explanatory variables	**Estimate**	**Odds ratio**	**95% CI for odds ratio**	**P value**
Transient response detected	-2.076	0.125	0.059 – 0.267	**<0.001**
Total number of captures (log transformed)	1.070	2.915	1.299 – 6.542	**0.009**
Age at detection of seropositive response (cub or adult)	0.434	1.543	0.733 – 3.250	0.253
Sex	-0.084	0.920	0.450 – 1.882	0.819

Model deviance = 44.1, df = 4, p<0.001

However, the likelihood of detecting a transient serological response was not significantly associated with either the age of the badger or whether there were infected reproductively active females resident in the social group of origin of the cub (model deviance = 2.3, df = 3, p=0.506).

4.4 Discussion

Social behaviour and the spatial organisation of wild animal populations can have a significant influence on the distribution of infection (Cross *et al.* 2009). In addition, certain individuals in a population may play a disproportionately important role in both the transmission and maintenance of infection (Lloyd-Smith *et al.* 2005). The purpose of this chapter was to use empirical data to determine whether the presence of certain individuals in social groups influences the likelihood of *M. bovis* acquisition and disease progression in badger cubs. Such information is of value in the development and implementation of future disease control strategies.

Broadly speaking, this study demonstrated significant associations between the disease status, sex and reproductive status of adult females in a social group, and both the acquisition and progression of disease in cubs, and their subsequent survival to adulthood. Furthermore no evidence was found to support any protective effect of transfer of maternal antibody to cubs.

In the present study, it was hypothesised that the greatest risk to cubs would be associated with the presence of infected breeding females due to their prolonged, repeated and intimate contact whilst cub immune systems are still maturing (Daruna 2004, Day 2007). Within this category it was anticipated that the level of risk to cubs would be higher where excretor females were present compared to seropositive females. A lower level of risk was predicted if the infected adults within the group were non-breeding females or adult males, as they would be expected to be in less intimate and prolonged contact with cubs. Within these groups, the higher risk would be likely to be associated with the excretors. The groups presenting the lowest risk for cubs were predicted to have resident adults with no evidence of infection (as measured by detection of a serological response or *M. bovis* excretion). Just under half of the cub cohorts in the study period were born into negative groups (Figure 4.1), representing approximately 35 cubs per annum. In contrast, the highest risk groups represented just 9% of the total number of group cub cohorts, equating to approximately 7 cubs per annum, which would have been exposed in the natal period to breeding females with evidence of *M. bovis* excretion. This suggests that only a relatively small proportion of the annual cohort of cubs were born to and reared by females excreting *M. bovis*. The remainder were exposed to adults with either less advanced stages of disease, or a reduced likelihood of early pre-weaning contact.

This proportional breakdown of social group infection status was however, constrained by the properties of the diagnostic tests underpinning the classification of the adult

badgers. Both the Brock ELISA and culture of clinical samples are insensitive tests for the detection of infection (Pritchard *et al.* 1986, Chambers *et al.* 2002, Chambers *et al.* 2009). However, progression of infection increases the sensitivity of the Brock ELISA (Chambers *et al.* 2009), as does repeat testing of individuals, but an uneven distribution of total captures amongst individual badgers makes it unwise to generalise as to the magnitude of this effect. Despite multiple test events, it is still highly probable that the 46% of social group-years being categorised as negative (Figure 4.1) is an overestimate, and that the proportions of social group-years categorised as infected are an underestimate.

Cubs born into groups with breeding excretor adult females were the most likely to be detected as both seropositive and excreting *M. bovis* in their first year (Tables 4.1 and 4.2). These findings are consistent with the hypothesis that the infectiousness of breeding females and the nature of their contact with their cubs present the highest risk of both the acquisition and progression of infection in cubs. Detection of *M. bovis* infection was based on the detection of a serological response. Poor sensitivity of the Brock ELISA limits the value of using the detection of a serological response as a proxy for infection, but this limitation applied equally to all badgers for a single test. The effect of multiple tests on an increase in sensitivity, which may not have applied equally to all badgers, was controlled for by including the number of test events as a co-variate in the analysis.

With respect to analyses examining the likelihood of the progression of infection to the point of *M. bovis* excretion, it may simply be that infection was acquired earlier in the cub's life. However, due to the chronic nature of tuberculous infections and the low likelihood of detecting cubs as excreting (CSL 2007), a more plausible explanation may be that it was due to a high infectious dose from an infectious dam to its cub at an age when the immune response was still maturing. The likelihood of severe progressive disseminated disease due to *M. tuberculosis* is highest in humans under 2 years of age, with particularly high mortality rates for children under 1 year of age (Cruz & Starke 2007). Without specific data on the development of a badger cub's immune response, this possibility remains speculative, but given the reported immunological similarities between dogs and badgers (Dalley *et al.* 2004), it is not unreasonable to assume that badger cubs, in line with puppies, are born with all the structural components of an immune system, the functionality of which matures during the first year of life (Day 2007).

The results of the present study build on previous research from the same population reporting an increased risk of disease detection in cubs in association with the presence of an adult excretor female in a group (Delahay *et al.* 2000a). However this present study goes

further in identifying evidence of a potential risk gradient in association with the characteristics of adults resident within the social group. The highest risk of acquiring infection and the highest risk that the infection would be rapidly progressive was found to be associated with cubs being born and reared in social groups with breeding females with evidence of *M. bovis* excretion, which is consistent with the prolonged, intimate and close contact a female badger would have with her cubs. Furthermore, these results add weight to findings from Chapter Three, that the detection of excretion by clinical sampling in the live badger, albeit of low sensitivity as a diagnostic test for infection (Pritchard *et al.* 1986, Chambers *et al.* 2002), is a useful tool for the identification of individuals of epidemiological importance in the population.

Experimental vaccination with Bacillus Calmette-Guérin (BCG) has been shown to reduce the progression and severity of *M. bovis* infection in captive badgers, and the likelihood of seroconversion in free-living badgers (Chambers *et al.* 2011). There is no evidence of a therapeutic effect of BCG vaccination in humans (Turner *et al.* 2000), hence the success of a strategy to vaccinate badgers may, in part be constrained by their prior infection status. Badger cubs begin to regularly emerge from the sett on average in late April/early May in the south of England (Neal & Cheeseman 1996). Before this occurs, badger cubs are unavailable for vaccination using current methods of delivery, hence if they are infected while still underground, it will not be possible to directly administer vaccine for prophylactic purposes.

During the study period 184 cubs, or 10% of the 1761 cubs captured, were detected as seropositive at some point during their first year. Due to the poor sensitivity of the Brock ELISA (range 37-53%) this will be an underestimate, although on average all cubs were tested twice, which would have the effect of reducing the number incorrectly classified as negative. In addition, seroprevalence fluctuates on an annual basis, and, despite relative stability in seroprevalence of about 10% from 1982 to the late 1990s, an increasing trend was observed from the late 1990s (CSL 2007). Hence there is some uncertainty surrounding the validity of only 10% of cubs being detected as seropositive during their first year. However, it seems reasonable to suggest that the majority of badgers were unlikely to have seroconverted until adulthood. Hence, cage trapping and vaccinating at any point following emergence and thereafter until the start of the closed season in the following February may target a large proportion of cubs prior to infection, although intuitively, the sooner cubs are vaccinated post-emergence the better.

There will, however, be a proportion of cubs that become infected pre-emergence and hence would not benefit regardless of how early trapping and vaccinating were implemented. It was not possible to estimate this proportion with the data currently available, but of the badgers that were detected as infected as cubs, just under half (42%) were detected at their first test event, and a large proportion of these took place in May and June (Figure 4.2), consistent with infection acquisition in these cubs at or close to the time of emergence. Once again, this proportion is almost certainly an underestimate due to the poor sensitivity of the Brock ELISA. The likelihood of test positivity at first capture was also significantly associated with the infection status of the social group, such that those cubs born into breeding excretor groups were the most likely to be positive at first capture (Figure 4.3, Table 4.3), and consistent with this finding there was a clustering of the positive test events for these particular cubs in May and June (Figure 4.4). There is therefore a small proportion overall of the cub population that are likely to be infected at or close to emergence and these cubs are likely to be spatially clustered in social groups with infectious breeding females. The following chapter will aim to address the limitations of these analyses associated with the low sensitivity of the Brock ELISA, by using data from the recently introduced and more sensitive gamma-interferon assay.

The likelihood that a cub would survive to adulthood was significantly lower in those individuals born into groups with breeding excretor females compared to cubs born into negative groups (Figure 4.4, Table 4.4). It is not unreasonable to propose that the poor cub survival observed in groups containing infectious breeding females was a direct consequence of disease induced mortality. Previous analyses have reported enhanced mortality in badgers that had been detected as excreting *M. bovis* (Wilkinson *et al.* 2000). Sample sizes and limitations of using only cub captures precluded statistical analysis of the data at this level, but the proposed mechanism of pseudo-vertical transmission with high infective doses over a prolonged period in immunologically immature cubs would be likely to put cubs into this advanced stage of disease during their first year of life.

Other factors may also contribute to cub mortality, for example, poor rearing and poor lactation in the excretor females. Mean cub body condition was, however, not a significant factor suggesting that the excretor females in these analyses were able to lactate and rear cubs just as effectively as non-excretor females.

The male biased mortality reported here is consistent with previous reports from this population (Cheeseman *et al.* 1987), and a suburban population in Bristol (Harris & Cresswell 1987). In addition, male biased juvenile mortality has been reported from red

deer populations and attributed to an enhanced susceptibility of males to food shortage associated with faster growth rates and greater nutritional requirements (Clutton-Brock *et al.* 1985).

One particular aim of the present study was to examine the data for evidence of any protective effect of maternally derived antibody as hypothesised in Newell *et al.* (1997). The findings however, suggest the opposite, in that there were significantly greater infection and disease progression risks in cubs born into infected groups, particularly those with infected reproductively active female badgers, and thus no evidence for any protection.

Results suggested that there was indeed a significant association between a transient seropositive response and a lower likelihood of later disease progression to excretion (Table 4.5). However, there were no significant differences in the likelihood of detection of this transient seropositive response between either cubs or adults, or between badgers born into groups with reproductively active infected females and those born into groups with no reproductively active infected females. Together these findings suggest that a transient response as described is not a valid proxy for maternally derived antibody as it is seen just as frequently where maternal antibody is highly unlikely to be the mechanism (i.e. in adults, and in cubs from groups with no reproductively active infected female badgers captured). From current understanding of the nature of successful immune responses to mycobacterial infections, it is unlikely that this transient response is in itself protective (Flynn & Chan 2001), and it is more biologically plausible that the appearance of a transient serological response in this dataset may arise from a false positive test result. Hence a high proportion of badgers with transient serological responses may not be truly infected, and will therefore have a lower likelihood of future *M. bovis* excretion.

As with any observational study of wildlife populations, there are assumptions that underpin some of the analyses presented here. The poor sensitivity of the diagnostic tests used (namely the Brock ELISA and mycobacterial culture of clinical samples) represent the most significant limitation and this has been discussed in the relevant sections. In addition, the categorisation of social group infection status was based on captures of adults and there was an assumption that the group of capture was a badger's resident group. It is possible, however, that an adult badger could be captured in different social groups in any given year, in which case it would have contributed to the infection status of more than one social group in that year. With respect to parentage of cubs, it was assumed that the reproductively active females in a group were in close contact with all of the cubs,

regardless of specific parentage. Without genetic data (which is likely to be forthcoming in future work), this is an unavoidable assumption, although there is some evidence for alloparental care in badger social groups, with females other than the dam being involved in cub rearing and even suckling (Woodroffe 1995).

In summary, these findings provide further evidence of the importance of social structure in wildlife populations. Over half of the group cohorts of cubs observed during this study period were reared in social groups with evidence of infection in the adult residents. The greatest risk of detection of infection was seen in cubs from groups with breeding excretor females, and in addition it was in these cubs that there was the highest likelihood of *M. bovis* excretion being detected, the highest over-winter mortality, and the highest likelihood of pre-emergent infection. However, cubs from these breeding excretor groups represented a small proportion of the total cubs born during the study period. These findings therefore provide strong evidence for an early and rapidly progressive disease picture associated with pseudovertical transmission, and for the importance of infectious breeding females in maintaining infection within a social group, and therefore within a population. Any onward contribution of infected cubs to transmission of infection within the population, in particular of those that survive to adulthood is outwith the scope of these analyses, but other studies assessing contact rates between individuals may shed further light on their relative importance.

With respect to potential vaccination strategies, there was no evidence of infection acquisition (as detected by seroconversion) in the majority of cubs emergent in the population, indicating that vaccine deployment in cubs at any time would be worthwhile, but intuitively the earlier this could be achieved the better. However, it should be noted that of the badgers that acquired infection as cubs, there was a proportion (albeit small and spatially clustered), that were probably infected prior to or close to the time of emergence. Using current methods of vaccine deployment (i.e. trapping and parenteral administration) it would not be possible to vaccinate those cubs prior to infection acquisition.

CHAPTER FIVE: Correlates of cell-mediated responses in badgers infected with *M. bovis*

5.1 Introduction

Mycobacterium bovis is an intracellular parasite. As such, a successful host response is likely to be characterised by a strong cell-mediated component of the adaptive immune response (Janeway *et al.* 2005). It is widely accepted that gamma-interferon (IFN), a cytokine associated with the cell-mediated response, is essential for protection from tuberculosis (Cooper *et al.* 1993, Flynn *et al.* 1993), although there is no simple correlation between IFN levels and protection (Fletcher 2007). Early experimental work in badgers detected an initial cell-mediated response following infection with *M. bovis* with no detectable serological response, followed by fluctuation in the cell-mediated component, and subsequently only a serological response, associated with the end-stages of infection (Mahmood *et al.* 1987b). This supports the hypothesis that a failing cell-mediated response with a reciprocal rise in the serological response is associated with the progression of infection, a hypothesis supported by work on *M. bovis* infection in cattle (Ritacco *et al.* 1991, Welsh *et al.* 2005) which can be illustrated in a conceptual model (Figure 5.1).

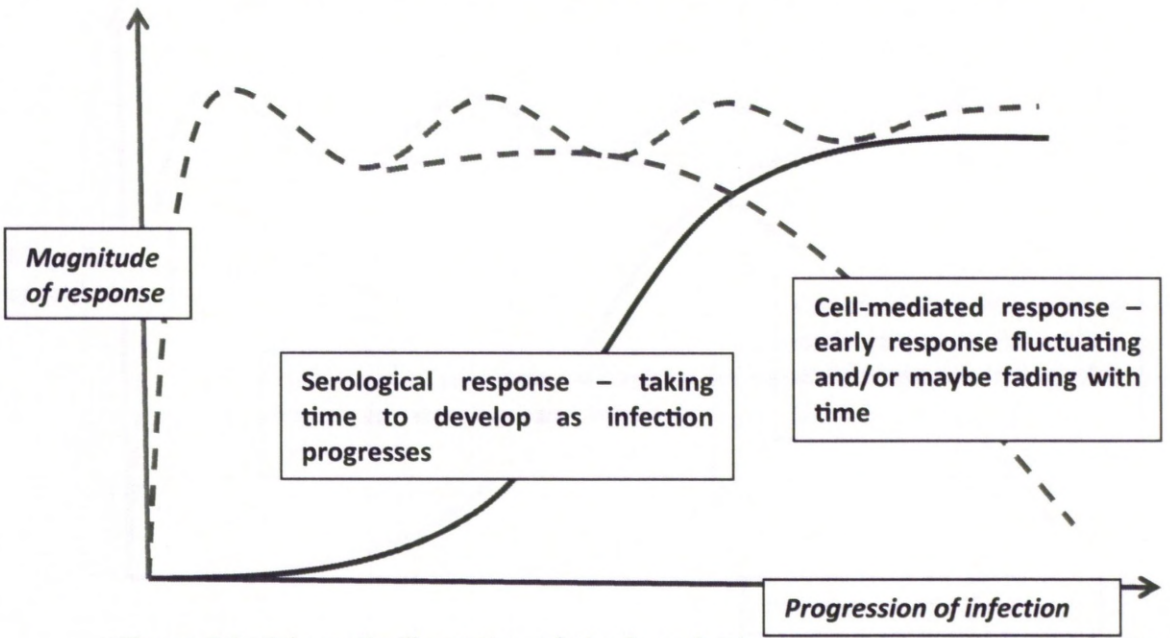

Figure 5.1. Schematic illustration of the hypothesised temporal progression of immune responses in badgers infected with *M. bovis*. Solid line indicates serological response; dotted line indicates cell-mediated response.

Diagnosis of bovine tuberculosis infection in the live badger is based on detection of specific immune responses and/or culture of *M. bovis* from sputum, faeces, urine, and swabs from bite wounds or abscesses. Serological responses to a single dominant antigen (MPB83) have historically been detected using the indirect Brock ELISA test (Goodger *et al.* 1994), and more recently in combination with other antigens in a rapid lateral flow immunoassay using the Brock TB StatPak test (SP) (Chambers *et al.* 2008). In a recent validation study of test performance, SP sensitivity was estimated at 49%, and specificity at 93% (Chambers *et al.* 2008). Following developments in cattle diagnostics, tests to detect IFN responses in badgers have been developed. Evaluation of the IFN assay estimated specificity at 93.6%, and sensitivity at 81% (Dalley *et al.* 2008). A recent analysis of age-related performance of the IFN assay reported a non-statistically significant lower sensitivity of 57.1% in cubs, compared to 84.6% in adults, leading the authors to suggest that a lower cut-off should be used in cubs in order to increase sensitivity to 71.4% while maintaining specificity at 95.0% (Chambers *et al.* 2009). IFN responses are measured in optical density (OD), in response to incubation with purified protein derivative from *M. bovis* (PPD-B), and purified protein derivative from *M. avium* (PPD-A). The results at a binary level are derived from the mean OD value after incubation with PPD-B minus the mean OD value after incubation with PPD-A, provided positive and negative control responses are within set limits (Dalley *et al.* 2008). OD levels above a specific cut-off value are deemed positive. Utilising the OD values in response to PPD-A improves the specificity of the assay by controlling for responses to environmental mycobacteria in the *M. avium* complex. Both the SP and IFN assays were introduced to the suite of diagnostic tests used at Woodchester Park in July 2006.

The Woodchester Park sampling protocol permitted sequential observations of immune responses in individual free-living, naturally infected badgers. In addition, the quantitative nature of the results from the IFN test allow temporal changes in the magnitude of the cell-mediated response to be observed and related to other test responses and other factors such as age and sex.

Early cell-mediated responses have been shown to be positively associated with the infective dose of *M. bovis* in experimental infection studies in cattle, possums and badgers (Buddle *et al.* 1994a, 1994b, Lesellier *et al.* 2009). One aim of this study was therefore to estimate the predictive value of the magnitude of the IFN response at its first detection, with respect to subsequent disease progression.

The conceptual framework (Figure 5.1) is largely based on experimental *M. bovis* infection studies. A specific aim of this study in naturally infected free-living badgers was to describe the temporal progression of the IFN response, in association with badger age, sex, and the stage to which disease subsequently progressed.

A further aim was to add to the findings from the previous chapter, which examined the risks of cub infection relative to the infection status of adults resident in the social group, using the Brock ELISA. This chapter describes a similar approach, but based detection of infection on the more sensitive IFN assay, albeit on a much smaller dataset.

5.2 Methods

The gamma interferon (IFN) and the Stat-Pak (SP) assays, were introduced to the diagnostic testing protocol at Woodchester Park in July 2006. Results from both tests, together with life-history and culture data from the same period were used in these analyses. In addition, historical test status data relating to *M. bovis* excretion and adult female reproductive activity prior to July 2006 were used in the categorisation of social group infection status. Life-history and test result data were collected as described in Chapter Two, General Methods. Results from the Brock ELISA were not used in these analyses, due to concerns over apparent changes in the performance of the test in this population since 2006. Seropositivity in this chapter therefore refers solely to results from the SP assay.

The OD values from the IFN assay were used in several of the following analyses. For reference, 'B-A' refers to (PPD-B mean) minus (PPD-A mean), or the difference in responses to bovine and avian mycobacterial antigens. 'Response to PPD-B alone' refers to ((PPD-B mean) minus (no antigen background mean)), and 'response to PPD-A alone' refers to ((PPD-A mean) minus (no antigen background mean)).

5.2.1 Age effects on test performance

On the basis of previous observations of differing IFN test performance in cubs and adults (Chambers *et al.* 2009), the IFN results from cub captures (badgers in their first year of life) were reclassified, using the optical density (OD) value of the difference between the average responses to PPD-B and PPD-A, to take account of the lower sensitivity reported in cubs. This involved using a cut-off of 0.023 instead of the standard 0.044.

To assess the difference between cub and adult responses to PPD-A alone, and how this may contribute to the differing test performance observed, a GLMM was constructed for PPD-A alone (log transformed) as the response variable, with age (cub or adult), and sex as explanatory variables, and individual badger fitted as a random effect. A second GLMM investigated the temporal progression of IFN responses to PPD-A alone during a cub's first year (log transformed), with the number of days from the start of the year, and sex as response variables, and individual badger fitted as a random effect.

5.2.2 IFN magnitude at incident event

In order to assess the potential predictive value of the magnitude of the IFN response at first detection with respect to subsequent infection progression, the dataset was

restricted to maximise the likelihood that the IFN detection event was as close to true IFN positive incidence as possible. This involved only including badgers with sufficient evidence from other tests (SP and culture) of the absence of infection prior to and at the detection event of the IFN response. In addition, badgers that were detected as IFN positive at their first test event were excluded.

Values of B-A at the incident event were plotted separately for badgers in which no evidence of subsequent infection progression was detected (no seropositive or *M. bovis* positive results), and badgers with evidence of subsequent infection progression (as determined by either a seropositive response or positive *M. bovis* culture).

A GLM was constructed to assess the predictive value of the magnitude of the IFN response at the incident event, as measured by B-A (log transformed) in relation to the maximal infection status acquired by each badger as defined above, whilst controlling for sex. Effects of the change in cut-off that had been applied to cubs, on the magnitude of B-A were controlled for by including age (cub or adult), as an independent factor in the model.

A second GLM was constructed to assess the predictive value of the magnitude of the IFN response at the incident event as measured by the response to PPD-B alone (log transformed). Explanatory variables were the maximal infection status acquired by each badger as defined above and sex. Age was also included in the model to control for anticipated differences between cub and adult responses to PPD-B.

5.2.3 Temporal progression of IFN responses

To examine how IFN responses may have changed over time in an individual, values of B-A at and subsequent to the incident event were plotted separately for badgers in which no evidence of subsequent infection progression was detected (no seropositive or *M. bovis* positive captures), and badgers with evidence of subsequent infection progression (as determined by either a seropositive response or positive *M. bovis* culture).

Three GLMMs were constructed, each of which included a different variable to describe the IFN response. These response variables were the test result (negative or positive), the magnitude of the response indicated by B-A (log transformed), and the responses to PPD-B alone (log transformed). Explanatory variables in the models were sex, maximal test status acquired (as defined above), and the time lapsed from the incident event to the test event (log transformed), with individual badger fitted as a random effect. Badger age at the test event (cub or adult) was included in all models to control for anticipated differences between the IFN responses of cubs and adults.

5.2.4 Correlates of cell-mediated and serological responses in cubs

The original dataset was restricted to badgers that were first captured and tested as cubs during the study period (July 2006 to January 2010 inclusive). This included some badgers that were test positive at their first capture event, and were hence excluded from the previous analyses.

In an approach similar to that used in the previous chapter, binomial regression was used to assess whether the probability of detecting infection in a cub was correlated with the infection status of the adult badgers resident in the cub's social group. Three categories of social group infection status were created using data from capture events for all badgers from each social group for the period May to the following January inclusive of each year. 'Negative' groups were those in which there no adult badgers that had been detected as excreting *M. bovis* were captured. 'Excretor' groups were those in which at least one adult badger with previous or current evidence of *M. bovis* excretion was captured, excluding reproductively active females (regardless of SP or IFN status). 'Breeding excretor' groups were those in which at least one reproductively active female with previous or current evidence of *M. bovis* excretion was captured (regardless of SP or IFN status).

The infection status of a social group effectively represented the natal environment for the cohort of cubs from that group. The frequency and proportion of social group years that fell into each category was summarised to illustrate the relative numbers of group cub cohorts that were likely to be exposed to infectious adults.

A GLM was constructed to investigate whether the likelihood of being detected as IFN positive as a cub was correlated with the infection status of the social group (as described above), whilst controlling for cub sex and the total number of cub test events (log transformed).

A second GLM was constructed to investigate whether the likelihood of being detected as seropositive as a cub was correlated with the infection status of the social group (as described above), controlling for cub sex, and the total number of cub test events (log transformed).

5.2.5 The timing of detection of IFN responses in cubs

The proportion of cubs that were detected as infected using the IFN test during that first year was reported for the whole study period, in conjunction with the mean number of test events for all cubs. A probability tree approach (as described in Forrester *et al.* 2001) was used to estimate the increase in sensitivity associated with multiple testing.

With respect to pre-emergent infection, assuming a perfect diagnostic test, cubs infected pre-emergence would test positive at the first event, which would also have to be reasonably close in time to emergence in late April/early May (Neal & Cheeseman 1996). Data were restricted to IFN positive cubs that were also tested before September, in order to balance being as close to emergence as practical, whilst at the same time permitting a reasonable sample size to test for associations. All test dates were standardised by conversion to an equivalent number of days from January 1st. A frequency distribution of the number of days from January 1st to the positive first test event was calculated. In addition, binomial regression was used to test whether the likelihood of pre-emergent infection was correlated with the infection status of the social group. A GLM was constructed using the likelihood of a cub's first capture event being IFN positive as a binomial response variable. Explanatory variables were the social group infection status and cub sex.

5.3 Results

5.3.1 Age effects on test performance

Following the adjustment of IFN cut-off values for 529 test events on cubs, 26 of 457 negative results were reclassified as positive. Of these, 23 were IFN incident events, increasing the number of IFN incident cubs from 47 to 70.

There was no significant difference between the magnitude of the responses to PPD-A alone for cubs and adults (Table 5.1). Furthermore, there was no significant association between the magnitude of the response to PPD-A alone, and the number of days from the start of the year for badger cubs (Table 5.1) although there was a non-significant trend such that responses increased over time during a cub's first year.

Table 5.1. Results from GLMMs investigating the difference between the responses to PPD-A alone for cub and adult badgers (n=1215 tests from July 2006 to January 2010), and secondly the temporal progression of responses to PPD-A during the first year of life (n=521 tests from July 2006 to January 2010).

Response variable	Explanatory variables	Direction of effect	Test statistic	P value
OD value of IFN assay in response to PPD–A alone (log transformed)	Age (cub or adult)	NA	$F_{(1,1212.0)} = 0.00$	0.992
	Sex	NA	$F_{(1,1212.0)} = 0.29$	0.592
OD value of IFN assay in response to PPD–A alone (log transformed)	Number of days from the start of the year	(Positive)	$F_{(1,484.5)} = 3.50$	0.062
	Sex	NA	$F_{(1,208.0)} = 0.84$	0.361

5.3.2 IFN magnitude at incident event

Following restriction of the dataset, there were 27 badgers with an IFN incident event that fulfilled the criteria for selection, with 82 post-incident capture events. There was a cluster of B-A values less than 0.2 for badgers with no evidence of infection progression beyond a positive IFN response. In contrast, the OD value of B-A ranged from 0.26 to 1.21 for four badgers with evidence of progression based on a subsequent SP positive test or detection of *M. bovis* excretion (Figure 5.2). There was one obvious outlier, a yearling male

(ID: 063Y) that was captured five times subsequent to its IFN incident event, but never detected as SP or culture positive (encircled in Figure 5.2).

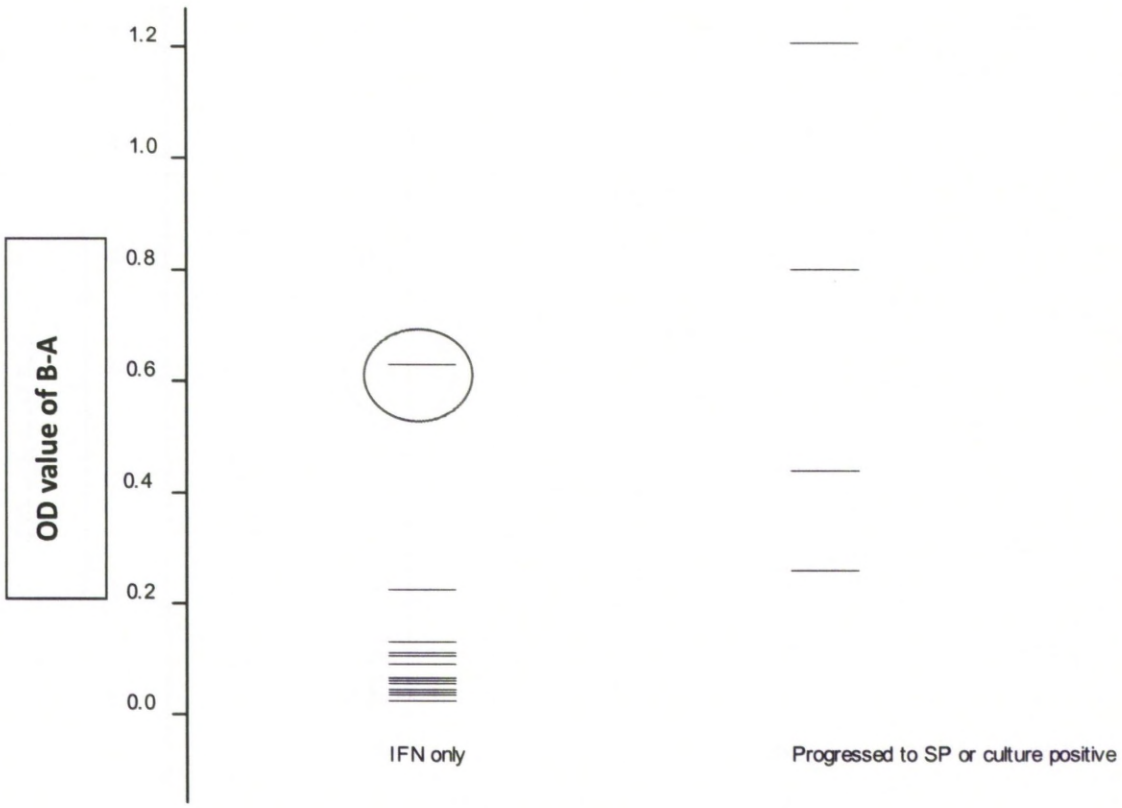

Figure 5.2. Magnitude (OD value) of B-A at IFN test positive incident event for 27 badgers that were subsequently detected as SP positive or excreting *M. bovis* (grouped together, n=4), and 23 that were not (n=23). Encircled is a yearling male (063Y).

The magnitude of the IFN response at the incident event, as measured either by the response to B-A or PPD-B alone, was significantly higher in badgers that were subsequently detected as having progressive infection as determined by either a serological response or the culture of *M. bovis* from clinical samples (Table 5.2). The magnitude of the IFN response to PPD-B alone was significantly lower in cubs than in adults, but there was no significant difference between cubs and adults for B-A. Sex was non-significant in both models.

Table 5.2. Results of GLMs to investigate factors associated with the magnitude of the incident IFN response in badgers (n=27 individuals captured from July 2006 to January 2010 inclusive).

Response variable	Explanatory variables	Reference level	Estimate	Test statistic	P value
OD value of IFN assay (B-A) at the IFN incident event (log transformed)	Maximal disease status = StatPak or *M.bovis*	IFN only	0.579	$t_{(23)} = 5.42$	**<0.001**
	Age (cub or adult)	Adult	-0.158	$t_{(23)} = -1.96$	0.062
	Sex	Female	-0.045	$t_{(23)} = -0.61$	0.550
	Final model $F_{(3,23)} = 14.84$, p<0.001				
OD value of IFN assay in response to PPD–B alone at the IFN incident event (log transformed)	**Explanatory variables**	**Reference level**	**Estimate**	**Test statistic**	**P value**
	Maximal disease status = StatPak or *M. bovis*	IFN only	0.930	$t_{(23)} = 5.23$	**<0.001**
	Age (cub or adult)	Adult	-0.392	$t_{(23)} = -2.92$	**0.008**
	Sex	Female	-0.098	$t_{(23)} = -0.80$	0.433
	Final model $F_{(3,23)} = 16.90$, p<0.001				

5.3.3 Temporal progression of IFN responses

For badgers with no evidence of infection progression beyond a positive IFN response, the majority of OD values for B-A remained below 0.2, following the IFN incident event (Figure 5.3). The OD values for the four badgers with evidence of progression, based on a subsequent SP positive test or detection of *M. bovis* excretion, were more randomly distributed. There were three clear outliers (Figure 5.3), which were IFN positive test results from the same yearling badger 063Y as previously identified (see also Figure 5.2). There were three further IFN negative captures for this individual in the following year, but at all six of its captures it was SP and culture negative.

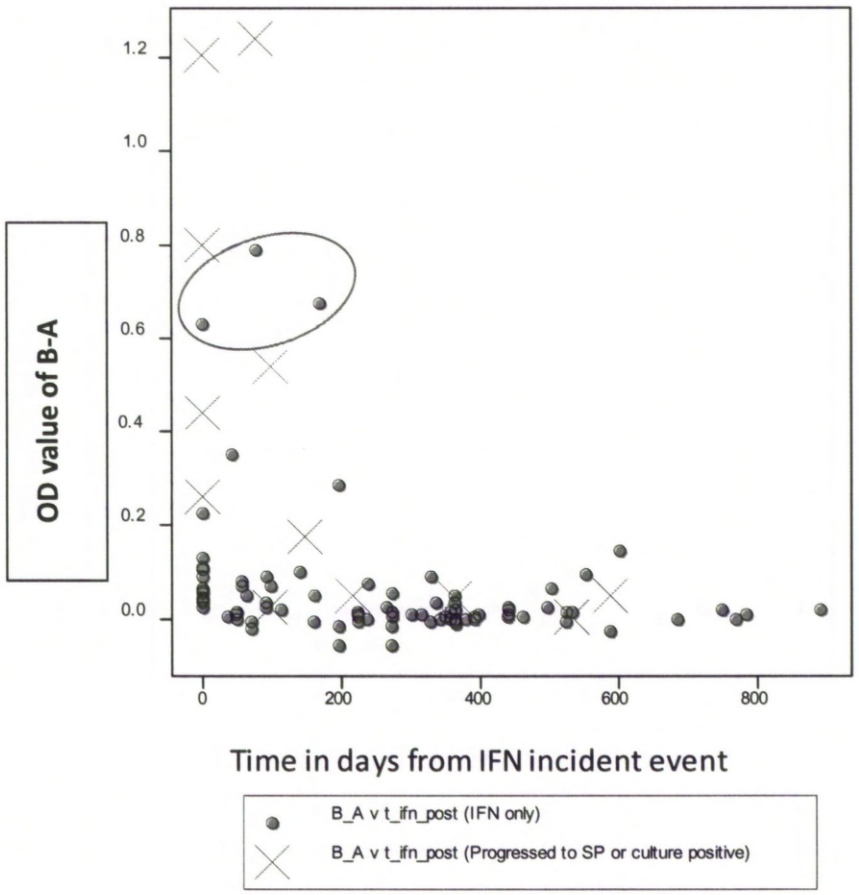

Figure 5.3. Temporal progression of IFN OD B-A values for 27 badgers using 27 incident and 82 post-incident capture events. Crosses represent captures from four badgers that were subsequently detected as either SP positive or excreting *M. bovis*. Small circles represent captures from 23 badgers that were subsequently negative to SP and culture tests. The large oval encircles three captures related to IFN positive events for 063Y.

The time period from the IFN positive incident event (log transformed), to when subsequent IFN tests were performed, was significantly negatively associated with the likelihood of an IFN positive test result or with the OD value of B-A, or in response to PPD-B alone. However, the likelihood of a positive result, or the OD value of B-A, or the OD value in response to PPD-B alone, was significantly higher in those badgers with evidence of disease progression (as detected by either a seropositive response or by *M. bovis* excretion) beyond an IFN response (Table 5.3). The magnitude of the IFN response in cubs was significantly lower than in adults for both the OD value of B-A, and in response to PPD-B alone, but at the binary test result level, there was no significant difference between cubs and adults. Sex was a non-significant factor in all the models.

Table 5.3. Results of GLMMs investigating factors associated with three measures of IFN response in badgers (positive/negative; OD B-A; OD PPD-B alone) following the IFN positive incident event (n=82 capture events for 27 individuals from July 2006 to January 2010 inclusive). Individual badger was included as a random factor in all models.

Response variable	Explanatory variables	Direction of effect	Test statistic	P value
IFN test result at captures subsequent to the IFN incident event Binomial distribution	Maximal disease status	Positive *M.bovis*/Stat-Pak>IFN	$\chi^2_{(1)} = 4.05$	**0.044**
	Time from IFN incident event (log transformed)	Negative	$\chi^2_{(1)} = 5.94$	**0.015**
	Age (cub or adult)	NA (adult>cub)	$\chi^2_{(1)} = 1.55$	0.214
	Sex	NA	$\chi^2_{(1)} = 0.43$	0.512
OD value of IFN assay (B-A) at captures subsequent to the IFN incident event – log transformed	Maximal disease status	Positive *M.bovis*/Stat-Pak>IFN	$F_{(1,28.4)} = 7.80$	**0.009**
	Time from IFN incident event (log transformed)	Negative	$F_{(1,65.2)} = 15.99$	**<0.001**
	Age (cub or adult)	Adult>cub	$F_{(1,75.0)} = 4.05$	**0.048**
	Sex	NA	$F_{(1,22.8)} = 1.22$	0.280
OD value of IFN assay in response to PPD–B alone at captures subsequent to the IFN incident event – log transformed	Maximal disease status	Positive *M.bovis*/Stat-Pak>IFN	$F_{(1,27.1)} = 6.83$	**0.014**
	Time from IFN incident event (log transformed)	Negative	$F_{(1,63.0)} = 14.90$	**<0.001**
	Age (cub or adult)	Adult>cub	$F_{(1,73.4)} = 4.31$	**0.041**
	Sex	NA	$F_{(1,22.4)} = 0.77$	0.390

5.3.4 Correlates of cell-mediated and serological responses in cubs

Restriction of the dataset to include only those badgers that were tested as cubs during the study period resulted in 866 capture events from 241 individuals. For the period May 2006 to January 2010 inclusive, there were 100 social group-year combinations and a total of 241 cubs were captured and tested. For 74% of social group-years, evidence of excretion was absent in all captured adults (Figure 5.4). This approximated to 74 social group cub cohorts and 178 cubs over 4 years, or 45 cubs per annum. Only 6 social group years (6%) were represented by a breeding excretor group. This approximated to 6 social group cub cohorts and 14 cubs over 4 years, or 4 cubs per annum.

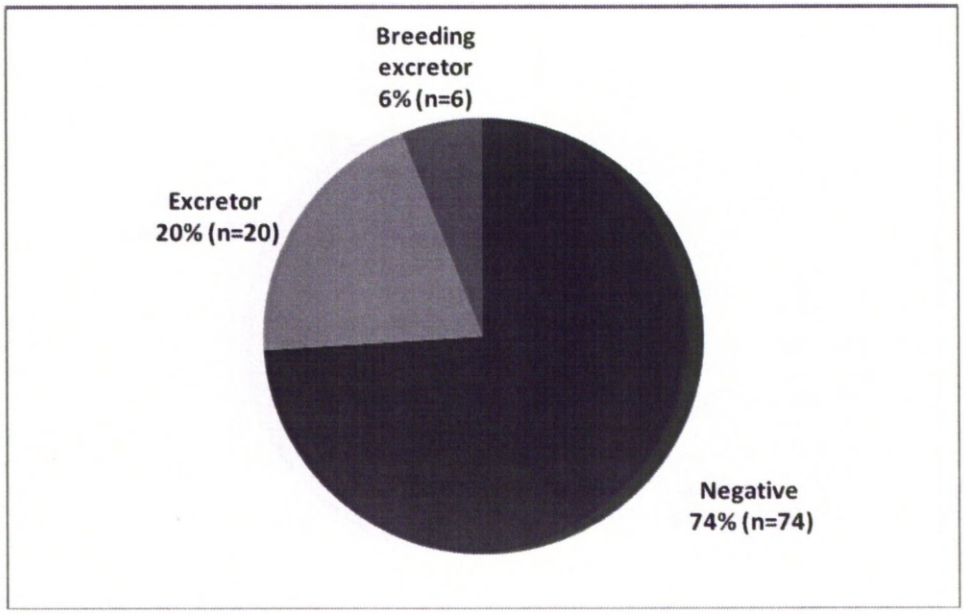

Figure 5.4. Frequency of occurrence of badger social group years falling into different categories of excretion status for the period May 2006 to January 2010 inclusive.

The likelihood of a cub being detected as IFN positive was significantly associated with its category of social group of origin, such that cubs born into breeding excretor groups were significantly more likely to be detected as IFN positive than cubs born into negative groups. There was a greater likelihood of cubs from excretor groups being detected as IFN positive as cubs than for those from negative groups, which was of borderline statistical significance (p=0.05) (Table 5.4). Sex was non-significant.

Table 5.4. Results from a GLM with a binomial distribution, investigating whether the likelihood of a badger cub being detected as IFN positive was correlated with social group infection status, whilst controlling for cub sex and the number of test events (n=241 cubs tested from July 2006 to January 2010 inclusive).

Variable	Reference level	Estimate	Odds ratio (OR)	95% CI for OR	P value
Excretor	Negative SG	0.662	1.939	1.000 – 3.759	0.050
Breeding excretor	Negative SG	1.875	6.522	2.407 – 17.67	**<0.001**
Total number of cub tests (log transformed)	NA	0.329	1.390	0.868 – 2.798	0.137
Sex	Female	0.444	1.559	0.720 – 2.683	0.327

Model deviance = 19.2, df = 4, $p<0.001$

The likelihood of a cub being detected as SP positive was significantly associated with the infection status of its social group, such that cubs born into breeding excretor groups were significantly more likely to be detected as SP positive than those born into negative groups. There was no significant difference in the likelihood of a cub being detected as SP positive relative to whether it born into an excretor group or born into a negative group (Table 5.5). Sex was non-significant.

Table 5.5. Results from a GLM with a binomial distribution, investigating whether the likelihood of a badger being detected as SP positive was correlated with social group infection status, controlling for cub sex and the number of test events (n=241 cubs tested from July 2006 to January 2010 inclusive).

Variable	Reference level	Estimate	Odds ratio (OR)	95% CI for OR	P value
Excretor	Negative SG	0.173	1.189	0.489 – 2.888	0.703
Breeding excretor	Negative SG	2.094	8.114	2.928 – 22.480	**<0.001**
Total number of cub tests (log transformed)	NA	-0.461	0.631	0.280 – 1.423	0.215
Sex	Female	0.467	1.595	0.763 – 3.334	0.267

Model deviance = 17.2, df = 4, p=0.002

5.3.5 The timing of detection of IFN responses in cubs

Of the 241 cubs tested using the IFN assay, 70 (29.0%, 95% confidence interval 23.4% - 35.2%) were positive. The mean number of test events for all 241 cubs was 2.2. Using a probability tree approach, and assuming independence of all tests, testing cubs twice increased the sensitivity (α), of detection of an IFN response from 71.4% (Chambers *et al.* 2009) for a single test to 91.8%, (α+ (α(1- α)), for two tests.

Of the 70 IFN positive cubs, 51 were first tested before September in their first year. Of these, 60.8% (95% confidence interval 46.1% - 74.2%) were positive at the first test. Most of these were captured in June, July and August (Figure 5.5).

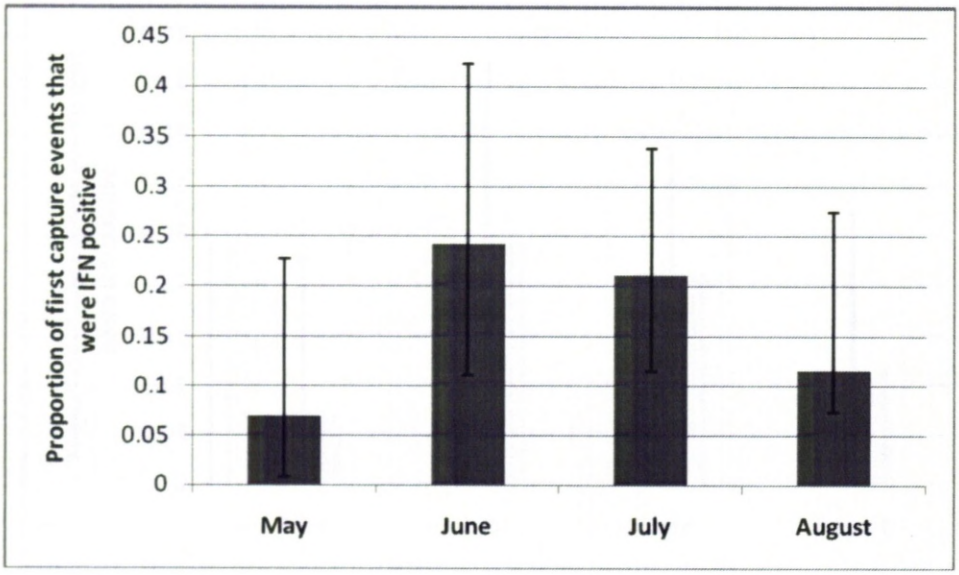

Figure 5.5. The proportion of first capture events that were IFN for badger cubs captured before September in their first year (2006 to 2010 inclusive). Error bars represent 95% CI.

There was no significant association between the category of social group of origin for IFN positive cubs and the likelihood of an IFN positive result at first capture (model deviance = 5.56, df = 3, p=0.135).

5.4 Discussion

The IFN and SP tests were only recently introduced at Woodchester Park, but the IFN test in particular offers advances not only in the diagnosis of *M. bovis* infection in the population, but in understanding the immunopathogenesis of natural *M. bovis* infection in free-living badgers. Furthermore, their introduction presented an opportunity to re-examine social group correlates of infection risk in cubs performed in the previous chapter using the less sensitive Brock ELISA and culture tests.

The principal aim of this chapter was to use quantitative data from the results of the IFN assay to assess the predictive value of the magnitude of the IFN response at the detection of the incident event, and its temporal progression in naturally infected wild badgers. A secondary aim was to investigate whether the risk of cub infection, as detected by both the more sensitive IFN, and SP assays, was correlated with the infection status of the cub's social group.

The magnitude of the IFN response to PPD-A alone did not differ between cubs and adults and did not increase significantly during a cub's first year of life (Table 5.1). These findings suggest that there is early exposure to environmental mycobacteria, and that cubs are able to mount a detectable IFN response to PPD-A that is lower, but not significantly so, than that seen in adults. It is therefore reasonable to propose that cub responses to PPD-A alone are not the main driver for the differing performance of the test in cubs and adults reported by Chambers *et al.* (2009), and it is likely that it is the response to PPD-B that is more important in this regard.

The magnitude of the IFN response at its first detection was significantly higher in badgers that were subsequently detected as either SP or culture positive (Table 5.2). In addition, although the likelihood of a positive IFN response and the magnitude of that response declined significantly with time, there was a greater likelihood of a positive response or a higher OD value in badgers with evidence of progression to a seropositive or culture positive state (Table 5.3). There was one obvious outlier badger, a male yearling at first IFN detection, which despite several subsequent captures was never detected as SP or culture positive, and there is no explanation from these data for its high IFN responses.

Although it is well accepted that cell-mediated immune responses, as measured by IFN levels for example, are necessary for protection from tuberculous infections (Cooper *et al.* 1993, Flynn *et al.* 1993), there does not appear to be any simple positive correlation between the magnitude of the IFN response and protection in either mice (Elias *et al.* 2005), cattle (Buddle *et al.* 2005), or humans (Doherty *et al.* 2002, Rook *et al.* 2005, Fletcher

2007). The findings in badgers reported here are consistent with a higher IFN response in association with more progressive infection. This could be related to either the infective dose of *M. bovis* or to the route of infection. A dose related response is supported by results from an experimental infection model, in which the magnitude of the cell-mediated response to PPD-B, as measured by a lymphocyte transformation assay, was positively correlated with the infective dose of *M. bovis,* as was the pathology observed in individual badgers (Lesellier *et al.* 2009). In addition, dose related responses to BCG have been reported in badgers using the ELISpot assay which measures the number of IFN producing T cells rather than the magnitude of the IFN response (Lesellier *et al.* 2011). Studies in cattle (Vordermeier *et al.* 2002, Buddle *et al.* 2005, Dean *et al.* 2005, Thacker *et al.* 2007) and humans (Doherty *et al.* 2002) provide further supportive evidence for a positive association between the magnitude of the IFN response and the severity of subsequent pathology.

It is possible that the route of infection may also be associated with higher IFN responses and more aggressive pathology. Evidence already exists suggesting that bite wounds in badgers may be associated with more aggressive pathology (Gallagher & Nelson 1979), and linking experimental intra-dermal injection of *M. bovis* with progressive systemic infection (Pritchard *et al.* 1987). Unfortunately no data on immune responses were collected in either of these studies. In the present study, there were only two badgers for which there was evidence of progression to *M. bovis* excretion. At the excretion incident events, one of the badgers yielded a positive culture solely from a bite wound on the rump, and one yielded a positive culture solely from a submandibular abscess with no recorded evidence of a bite wound. Unfortunately the very small sample size precludes drawing any conclusions.

The temporal reduction in the magnitude of the IFN response in badgers in the present study is the only study where the IFN response to natural *M. bovis* infection has been monitored over a prolonged period in a wildlife population. In those badgers with no evidence of progression of infection subsequent to a positive IFN test result, the magnitude of the IFN response at the disclosing event was relatively low and remained so. The observed reduction in the magnitude of the response over time could be consistent with either latent infection thereafter, with intermittent peaks of *M. bovis* replication and potentially concomitant peaks in IFN responses, or in badgers where the response faded completely, it could be related to full resolution and clearance of infection.

In contrast, in those badgers with evidence of progressive infection subsequent to a positive IFN test result, the magnitude of the IFN response at the disclosing event was

relatively higher and was maintained at a higher level, despite it also fading overall with time. The reduction in the magnitude of the IFN response over time could be consistent with either a gradual exhaustion of the ability to mount an IFN response in the face of antigenic stimulation, or with progressive anergy in response to continued antigenic stimulation. Skin test anergy has been reported in cattle (Lepper *et al.* 1977, Houlihan *et al.* 2008), and in humans (Maher *et al.* 1992), in particular in association with immunosuppression, HIV co-infection (Lalvani & Pareek 2009) and chronic renal failure (Segall & Covic 2010). Longitudinal data on IFN responses are scarce, but from a study in humans infected with *M. tuberculosis*, it appears that there may be some loss of sensitivity in the IFN assays that are based on measuring the concentration of IFN produced from blood cells stimulated with PPD-B (such as in the test used in these analyses) (Raby *et al.* 2008), but the assays that measure the number of IFN producing cells (so-called ELISpot assays) (Lalvani & Pareek 2009) appear to maintain sensitivity in the face of immunsuppression in association with HIV co-infection (Liebeschuetz *et al.* 2004).

The magnitude of the IFN response at the incident event may have some value as a predictor for disease progression in an individual badger. However, its practical value as a tool for the selective identification of individuals for management interventions is limited, not least because of the 9 hour incubation period required, and insufficient data to generate useful estimates of the confidence of predictions. As further data become available, it may be feasible to derive a cut-off, above which the probability could be estimated of an IFN incident badger being subsequently detected as SP positive, or as excreting *M. bovis* at live sampling.

The magnitude of IFN response to PPD-B was significantly lower in cubs than adults, both at and subsequent to the IFN positive incident event. Together with non-significant differences between cub and adult responses to PPD-A, it would seem that a lower response to PPD-B is the main driver for the reduced sensitivity of the IFN assay as observed in cubs (Chambers *et al.* 2008).

Interestingly the sex of the badger was non-significant in all the analyses suggesting that there is no detectable difference between the magnitude and temporal progression of IFN responses of males and females. This is in contrast to the observations that the time from single positive culture to continued excretion was found to be shorter in males than females (Chapter Three), and that male badgers had significantly higher mortality rates than female badgers following the detection of excretion (Wilkinson *et al.* 2000). It is also surprising in the light of similar findings in previous chapters of the present study, and

evidence of enhanced cell-mediated immune responses reported in female humans (Bouman *et al.* 2005).

It should be noted that the method of detection of IFN used in the present study involved cellular stimulation of peripheral blood cells, and was therefore testing an individual badger's ability to mount a response to specific stimuli (in this case, PPD-B and PPD-A) (Buddle *et al.* 2005). This approach is therefore not a direct measure of *in vivo* IFN levels at the site of bacterial multiplication, rather the degree to which the immune system has been primed to respond by the pathogen itself. Differences between IFN levels locally and in the peripheral blood have been detected in experimentally infected badgers (Lesellier, 2007), although in cattle there are data to suggest that immune responses measured in peripheral blood mirror those at the site of infection (Rhodes *et al.* 2000).

In addition, the periodic nature of the badger trapping events in the present study means that IFN responses may have in fact occurred prior to the disclosing test event. However, the rigorous restriction of the dataset ensured this effect was minimised as far as possible, by excluding badgers with evidence of infection (based on SP and culture tests) prior to and at the IFN incident event, and excluding those with no test history prior to the IFN incident event.

Previous analyses reported a positive association between the likelihood of infection with *M. bovis* (as detected by *M. bovis* excretion), and the presence of excretor status individuals within a badger social group (Delahay *et al.* 2000a, Vicente *et al.* 2007a). The analyses reported here enhanced chances of detection of infection by using the IFN assay, which is currently the most sensitive test of *M. bovis* infection in live badgers. In addition, it was possible to describe each social group in each year (May to December) in terms of the excretion status of resident adults within the group during the cub rearing period. Theoretically, reproductively active excretor females represent an elevated risk to young cubs with opportunities for disease transmission in association with lactation and prolonged and repeated periods of close social contact, before and following cub emergence above ground. This potential source of infection has been termed 'pseudo-vertical transmission' (Cheeseman *et al.* 1988). The potential for alloparental care (females caring for young that are not their own progeny, and possibly even lactating) in badger social groups has also been explored in previous studies (Woodroffe 1993). Such behaviour would provide enhanced opportunities for transmission from excreting females to cubs regardless of whether they were mother and offspring.

Of the 100 social group years in the study period, only six involved the presence of a reproductively active excretor female, and 20 included an excretor adult (excluding reproductively active females). In contrast, in 74 of the groups, there were no captures of adults of excretor status during the cub rearing period (Figure 5.4). Thus the majority of social group cub cohorts, during this study period, were born and reared in groups with no evidence of resident adults excreting *M. bovis*. Despite this, the likelihood of detection of an IFN or a SP response in a cub was significantly higher in groups with a reproductively active excretor female (Tables 5.4 and 5.5). This is consistent with the hypothesis that reproductively active females may be particularly important in the maintenance of infection within badger social groups. Although there was a similar general trend for the presence of any other badgers of excretor status in the social group, this effect only approached significance (Table 5.4), and for the SP response, there was no significant association (Table 5.5).

The categorisation of social groups based on the infection status of the adults captured was reliant on the detection of *M. bovis* excretion. Despite the increased sensitivity of detection associated with repeat captures of the same individuals, it is probable that, given the low sensitivity of culture from live sampling (Pritchard *et al.* 1986), the reported 74% of groups categorised as negative is an overestimate of the true proportion.

Vaccination with Bacillus Calmette-Guérin (BCG) has been shown to reduce the progression and severity of *M. bovis* infection in experimental studies with captive badgers, and the likelihood of seroconversion in naturally infected free-living badgers (Chambers *et al.* 2011). The success of a vaccination strategy would, in part, be constrained if cubs were infected prior to their availability above ground for vaccination. During the study period 70 cubs (29% of the 241 cubs captured) were detected as IFN positive at some point during their first year. This is likely to be an underestimate as no test is 100% sensitive, but an increase in sensitivity from 71% to 92% in association with a mean of two tests per cub suggests it is likely to be close to the true figure. It therefore seems reasonable to suggest that the majority of cubs did not acquire infection until adulthood. Hence, cage trapping and vaccinating at any point during their first year would be likely to be beneficial, although intuitively, the sooner cubs are vaccinated post-emergence the better.

In the present study, the majority (61%) of the IFN positive cubs that were tested prior to September were positive at first capture, but there was no significant association with the infection status of their social group, although sample sizes were small. However, only about a third of these events occurred before mid June, with the majority of cubs that were

IFN positive at their first test not being captured until well into July and August (Figure 5.5). Interpretation of these data is difficult, but they are consistent with a substantial proportion of the cubs detected as infected during their first year, being infected at or close to the time of emergence.

In summary, the findings from the present study are supportive of an initial cell-mediated response following *M. bovis* infection in badgers fading with time (see Figure 5.6). This has previously been reported in cattle (Ritacco *et al.* 1991, Welsh *et al.* 2005), but not from free-living naturally infected badgers. The magnitude of the IFN response at the incident event, and the rate of its decline may also be useful predictors of future disease progression within individuals. In addition, there is evidence that pseudo-vertical transmission contributes significantly to the transmission of infection to cubs and the maintenance of infection in the population. These findings, however, provide scant information about the relative contribution of cubs infected in this way to further onward transmission within the badger population. Application of these findings to other populations should be carried out with caution as prevalence and distribution of infection may vary amongst badger populations. Nevertheless, a strategy that aims to deliver vaccine as early in the year as practical appears likely to be beneficial to the majority of cubs, while fully accepting that there will be a small proportion of individuals that are infected too early to be accessible for vaccination.

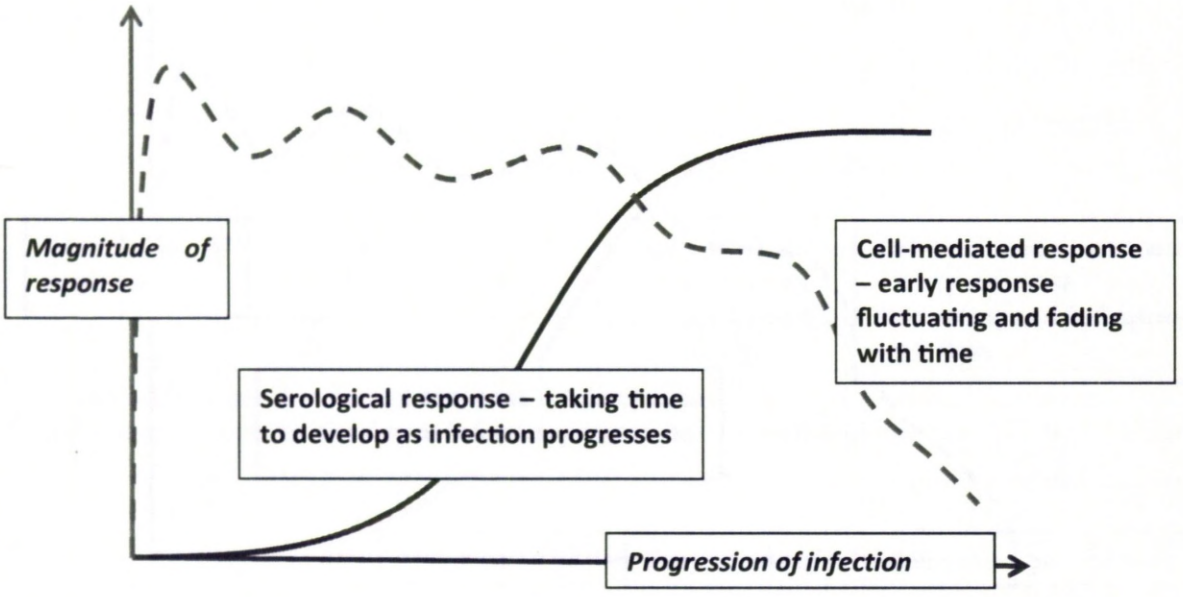

Figure 5.6. Conceptual model of the temporal progression of immune responses in badgers naturally infected with *M. bovis*. Solid line indicates serological response; dotted line indicates cell-mediated response.

CHAPTER SIX: Haematological, blood biochemical and parasitological correlates of *M. bovis* infection in badgers

6.1 Introduction

Fluctuating levels of nutritional, seasonal, reproductive and social stress are likely to be reflected in haematological and biochemical parameters in free-living wildlife. In addition, certain pathological conditions may be characterised by changes in haematology and biochemistry parameters, but it is not common for observed changes to be condition specific, although a combination of changes may be characteristic of a particular condition or disease. There are scant data on clinical pathology reference ranges for badgers, and in particular for wild badgers. Domingo-Roura *et al.* (2001) investigated seasonal variation of several parameters in a wild badger population, and Winnacker *et al.* (2008) reported ranges for haematology and blood biochemistry from the Woodchester Park population.

There is also limited published research on the haematological and biochemical changes associated with infection with tuberculous mycobacteria. In humans, mild anaemia, and other blood cell abnormalities in association with tuberculous bone marrow infiltration have been reported (Fountain 1954, Cameron 1974). Experimental *M. bovis* infections were associated with lymphocytopenia and eosinopenia in brushtail possums (*Trichosurus vulpecula*) (Buddle *et al.* 1994), and monocytosis and lymphocytosis in American bison (*Bison bison*), although values were still within normal limits for the species (Miller *et al.* 1989). In badgers, elevations in red cell counts have been reported by Chambers *et al.* (2000), but a more variable haematological picture was described by Mahmood *et al.* (1988), with decreases in red and white blood cell counts and haemoglobin concentrations in the later stages of disease.

The serum proteins, and in particular those associated with the acute phase response, have been the focus of research with respect to many infectious diseases in humans and domesticated animals (Cerón *et al.* 2005), and there is equivocal evidence for the use of C-reactive protein, a positive acute phase protein (APP), to differentiate tuberculosis from other infectious causes of respiratory disease (Choi *et al.* 2007, Breen *et al.* 2008). To date, their potential value in wild animal disease models has not been evaluated. Other blood biochemistry parameters have been associated with *M. bovis* infection in badgers. Elevations in creatinine and GGT, and reductions in calcium and bilirubin were reported by

Chambers *et al.* (2000). However, in humans, *elevated* calcium levels have been documented in association with tuberculosis (Chan *et al.* 1994).

Infection with tuberculous mycobacteria is characterised in many species by a complex host-pathogen interaction. One mechanism underlying this complexity is the effect of co-infections, in particular when there is mutual antagonism between the optimal immune responses for each pathogen (Fenton *et al.* 2008). This is a challenging and relatively poorly understood research area, and highlights the importance of longitudinal studies of naturally infected wild animals with multiple pathogens (Telfer *et al.* 2008), in contrast to single pathogen models common in experimental research.

The role of helminth infections and the potential for them to drive the immune response in a direction that is suboptimal for mycobacterial control has been the subject of work in humans infected with *M. tuberculosis* (Bentwich *et al.* 1999, Elias *et al.* 2001, Resende Co *et al.* 2006). In addition, in an experimental co-infection model of cattle with both *M. bovis* and *Fasciola hepatica*, the cell-mediated responses specific to *M. bovis* were reduced, and the severity of the tuberculous infection was greater (Flynn *et al.* 2009). In a study of African buffalo (*Syncerus caffer*) with endemic *M. bovis,* Jolles *et al.* (2008) speculated that the successful elimination of one pathogen at the expense of infection with another, and increased mortality in co-infected hosts, were associated with the observed negative correlation between *M. bovis* and helminth infections. The importance of parasite interactions in wild animal disease has been well documented in other disease models (Cox 2001, Bradley & Jackson 2008, Vicente *et al.* 2007b, Lello & Hussell 2008), but to date this has attracted little attention in studies of tuberculosis infection in wildlife.

Analyses in this chapter tested whether infection with *M. bovis* in badgers was correlated with several haematology and blood biochemistry parameters. The purpose of these analyses was firstly to detect disease-induced effects on physiological processes to gain insight into the impact of *M. bovis* infection at the individual level, and secondly to assess whether the parameters selected had any diagnostic value in the detection or progression of *M. bovis* infection.

Further analyses tested whether intestinal endoparasite burdens were correlated with badger body condition and the magnitude of the cell-mediated immune response to *M. bovis* infection. This was based on the hypothesis that Th_1 responses predominate in protozoal and mycobacterial infections, and Th_2 responses predominate in helminth infections (Fenton *et al.* 2008).

6.2 Methods

Six badger social groups were selected for more extensive data collection from May 2007 to January 2010. Selection was based on the criteria that there was evidence from captures during the previous 2-3 years of resident badgers excreting *M. bovis*. In addition to the routine observations and sampling protocol described in Chapter Two (General Methods), the presence of all bite wounds was recorded. Those that were open and discharging blood and/or pus were described as 'open fresh'. Those that were still open and healing, but where any discharge had largely resolved, were described as 'open old'. Where there was epithelialised tissue consistent with scar tissue and no evidence of current inflammation they were described as 'healed'. In addition, extra blood samples were taken for clinical pathology, and faecal samples were taken for estimation of intestinal endoparasite burdens.

Whole blood samples, collected into EDTA anticoagulant, a fresh air-dried blood smear and whole blood samples taken into plain tubes, were submitted to an external laboratory (Carmichael Torrance Diagnostic Services Ltd., Garforth, UK) for analysis. Haematological parameters measured were red blood cell count (RBC), packed cell volume (PCV), total haemoglobin (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), platelet count, total white blood cell count (WBC) and differential white blood cell count. Biochemical parameters measured were total protein, albumin, globulin, urea, creatinine, calcium, phosphorus, gamma glutamyl transferase (GGT), alanine amino transferase (ALT), alkaline phosphatase (ALP), total bilirubin, total cholesterol, low density lipoproteins, high density lipoproteins and triglycerides. In addition, serum protein electrophoresis (SPE) was used to partition the contribution of serum levels of alpha, beta and gamma globulins respectively to the total globulin fraction.

Faecal samples were collected as described in Chapter Two (General Methods), and a 4g aliquot was transferred into a separate sterile plastic pot. This was then mixed in a 1:1 ratio with 70% ethanol and refrigerated for 24 hours prior to faecal egg count analysis (FEC), to kill any *M. bovis* in the samples (Scanlon & Quinn 2000). Following 24 hours of refrigerated storage, 6g of the faeces/ethanol mixture were added to 39ml of tap water and shaken well with glass beads for 2 minutes to break up the faecal matter. The mixture was poured through a tea strainer and the debris in the strainer discarded. The filtrate was evenly mixed, and 15ml added to a centrifuge tube, which was then set to spin at 800G for 2 minutes. The supernatant was discarded and the pellet resuspended with 15ml of saturated salt solution. The tube was inverted to mix fully and a plastic pipette was filled

immediately from the middle of the tube and used to fill both chambers of a McMaster slide. After two minutes to allow full flotation, nematode ova and coccidian oocysts were counted in both chambers, differentiating to genus level or species level where possible. The total numbers of ova and oocysts counted were multiplied by 50 to give an estimate of the number of ova/oocysts per gram of faeces.

6.2.1 TB infection status

Primary or secondary infection status at each capture event was assigned using results from the gamma-interferon test (IFN), Stat-Pak test (SP), and mycobacterial culture from clinical samples for co-incident captures during the period May 2006 to January 2010 inclusive, and results from IFN, SP and culture from all previous captures prior to May 2007 where applicable (Table 6.1). Results from the Brock ELISA were, however, not taken into consideration due to concerns over test performance from 2006 onwards. Hence seropositive status arose solely from a positive SP result.

Table 6.1. Primary and secondary criteria, based on IFN, SP and culture test results up to and including the capture event in question, for assigning TB infection status to each badger at each capture event.

TB infection status		**Test results**
Primary infection status	Negative	All negative
	IFN	≥ one IFN positive, all captures SP and culture negative
	Seropositive or Excretor	≥ one SP or culture positive
Secondary infection status	Non-excretor	Culture negative, regardless of IFN and SP results
	Excretor	≥ one culture positive, regardless of IFN and SP results

6.2.2 Haematological and blood biochemical parameters as correlates of *M. bovis* infection

GLMMs were constructed separately for each haematological and blood biochemical parameter selected, to test whether they were correlated with the detection of *M. bovis* infection. In each model the response variable was the blood parameter, and the

explanatory variable primary infection status, as defined above (Table 6.1). Each model also controlled for season (Domingo-Roura *et al.* 2001), age (Domingo-Roura *et al.* 2001, Winnacker *et al.* 2008) and sex. Repeat captures of the same badgers were controlled for by including individual as a random effect. Seasons were categorised as follows: May as spring; June, July and August as summer; September, October and November as autumn; and December, January and February as winter.

Haematological, normally distributed response variables were RBC, WBC, Hb and lymphocyte counts. Monocyte and eosinophil counts were not normally distributed; hence they were recoded as binomial response variables, monocytosis and eosinopenia respectively, using cut-off values derived from the 95% confidence intervals for the mean values in Winnacker *et al.* (2008). Monocytosis was recorded for values in excess of 0.17 $x10^9$/l, and eosinopenia was recorded for values less than 0.001 x 10^9/l. Blood biochemical response variables were the serum protein concentrations, comprising total albumin, total globulin, alpha, beta and gamma fractions, and GGT, creatinine, calcium and bilirubin. All variables were selected based on previous reports (Mahmood *et al.* 1988, Buddle *et al.* 1994b, Chambers *et al.* 2000, Cerón *et al.* 2005).

An additional explanatory variable, the presence of active bite wounds, was included in the analyses involving the haematological parameters and the serum proteins, to control for variations associated with the non-specific inflammatory response to tissue trauma (Petersen *et al.* 2004). Only bite wounds recorded as 'open fresh' or 'open old' were classified as active. This factor was not included in the analyses of the other blood biochemical parameters since the parameters selected were unlikely to be associated with the non-specific inflammatory response, in the same way as haematological parameters and serum proteins (Kerr 1989).

6.2.3 Parasitological correlates of *M. bovis* infection

Previous studies have reported several common intestinal parasites of badgers in the UK that would be detectable by faecal examination. These included three species of nematode (*Molineus patens, Uncinaria stenocephala* and *Capillaria erinacei*) (Hancox 1980, Jones *et al.* 1980, Dale 2005), and two species of coccidia (*Eimeria melis* and *Isospora melis*) (Anwar *et al.* 2000). Prevalence and intensity data for the parasite ova/oocysts found in this study were summarised by age.

To assess associations between parasite burdens and the magnitude of the IFN response to *M. bovis* infection, the dataset was restricted to exclude captures of negative badgers as defined by the primary infection status (Table 6.1). Three GLMMs were

constructed using the presence of nematode ova, and of the two species of coccidia, as binomial response variables respectively, and three GLMMs were constructed using the intensity of those parasites respectively. Intensity was recorded as the number of ova/oocysts per gram of faeces and log transformed to normalise the distribution. Explanatory variables in each model were the magnitude of the IFN response as measured by the mean optical density (OD) value in response to PPD-B minus the mean OD value in response to PPD-A (Chapter Three), badger age (cub or adult), sex, and season, with individual badger fitted as a random effect. Seasons were categorised as previously described.

6.2.4 Parasite burdens and badger body condition

To assess associations between parasite burdens and the body condition of badgers, the standardised body condition index as previously described (Chapter Three) was used as the normally distributed response variable in GLMMs constructed separately for cubs and adults, and separately for the presence and intensity of parasite burdens. Explanatory variables were the presence or intensity (as previously described) of nematode ova, *Eimeria melis* and *Isospora melis* oocysts, excretion status (see Table 6.1), and sex, with individual badger fitted as a random effect. Excretion status was included to control for the association between excretion status and poor body condition previously reported (Chapter Three). This did not apply to the cub models as there were no culture positive captures of cubs during the study period. Tests for significant interactions between excretion status and each of the parasite factors were included in the adult models, but removed if non-significant.

6.3 Results

6.3.1 Haematological and blood biochemical parameters as correlates of *M. bovis* infection

The white blood cell count was significantly associated with primary infection status, such that the highest counts were seen in badgers in seropositive or excretor status and the lowest counts in badgers of negative infection status (Figure 6.1). Red blood cell counts and haemoglobin concentration were significantly higher in adults than cubs, and the values of red cell parameters and lymphocytes were highest in the winter months. In contrast, eosinopenia was significantly more likely to be detected in the winter months (Table 6.2).

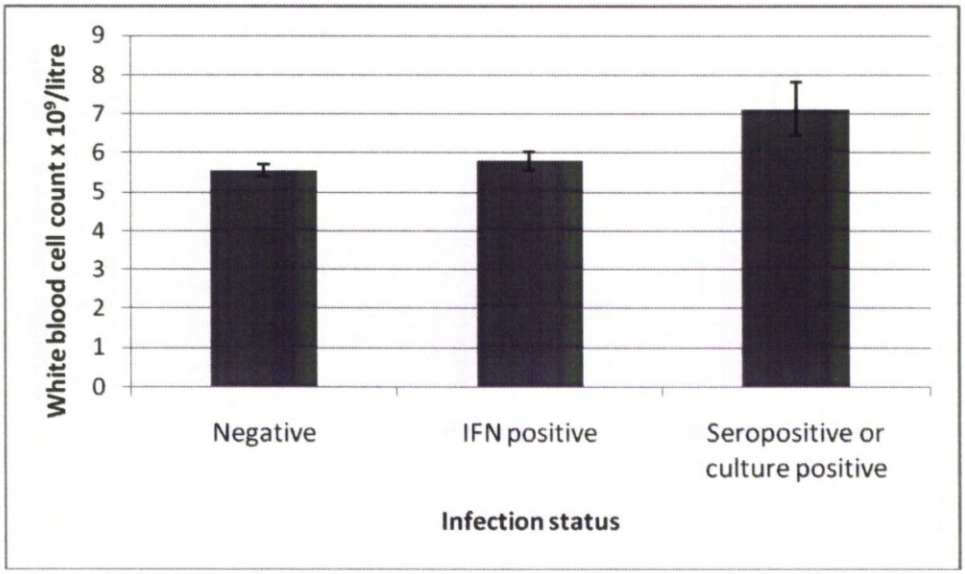

Figure 6.1 Mean white blood cell counts from 275 captures of 86 individual badgers of differing infection status from selected social groups (May 2007 to January 2010 inclusive). Error bars represent SEM. Overrall mean = 5.89 x 10^9/l.

Table 6.2. Haematological correlates of *M. bovis* infection in badgers using GLMMs, controlling for the presence of active bite wounds, season, sex and age, with individual badger fitted as a random effect in all models (n=275, May 2007 to January 2010 inclusive).

Response	Explanatory variables	Test statistic	P value
Haemoglobin g/dl	Primary infection status	$F_{(2,158.3)} = 0.05$	0.703
	Active bite wound	$F_{(1,246.4)} = 0.38$	0.541
	Season	$F_{(3,221.5)} = 34.26$	**<0.001**
	Sex	$F_{(1,72.2)} = 0.19$	0.662
	Age	$F_{(1,264.3)} = 159.12$	**<0.001**
RBC x10^{12}/l	Primary infection status	$F_{(2,144.3)} = 0.19$	0.829
	Active bite wound	$F_{(1,254.1)} = 2.04$	0.155
	Season	$F_{(3,232.1)} = 27.11$	**<0.001**
	Sex	$F_{(1,73.9)} = 0.07$	0.786
	Age	$F_{(1,265.7)} = 140.97$	**<0.001**
WBC x10^9/l	Primary infection status	$F_{(2,139.5)} = 5.45$	**0.005***
	***Predicted mean values. Negative = 5.99 x 10^9/l; IFN positive = 6.08 x 10^9l; Seropositive/excretor = 7.82 x 10^9/l**		
	Active bite wound	$F_{(1,257.3)} = 1.58$	0.210
	Season	$F_{(3,236.4)} = 2.61$	0.052
	Sex	$F_{(1,75.4)} = 0.34$	0.562
	Age	$F_{(1,264.7)} = 1.97$	0.162
Lymphocyte count x10^9/l	Primary infection status	$F_{(2,120.9)} = 2.86$	0.061
	Active bite wound	$F_{(1,263.3)} = 0.33$	0.567
	Season	$F_{(3,244.5)} = 3.76$	**0.011**
	Sex	$F_{(1,73.5)} = 0.65$	0.422
	Age	$F_{(1,259.2)} = 0.02$	0.899
Monocytosis (values >0.17 x 10^9/l)	Primary infection status	$\chi^2_{(2)} = 0.21$	0.814
	Active bite wound	$\chi^2_{(1)} = 1.19$	0.275
	Season	$\chi^2_{(3)} = 1.38$	0.248
	Sex	$\chi^2_{(1)} = 0.13$	0.723
	Age	$\chi^2_{(1)} = 0.00$	0.962
Eosinopenia (values <0.001 x 10^9/l)	Primary infection status	$\chi^2_{(2)} = 0.52$	0.594
	Active bite wound	$\chi^2_{(1)} = 1.84$	0.175
	Season	$\chi^2_{(3)} = 8.19$	**<0.001**
	Sex	$\chi^2_{(1)} = 0.03$	0.858
	Age	$\chi^2_{(1)} = 0.38$	0.539

The concentration of the gamma globulin fraction was significantly associated with infection status, such that the highest concentration was observed in badgers of seropositive or excretor status, and the lowest in badgers of negative status (Figure 6.2 and Table 6.3). In addition, the gamma globulin concentration was significantly higher in badgers with active bite wounds. The concentration of all the serum proteins was highest in the winter months, and was significantly higher in adults than cubs with the exception of the alpha fraction for which there was no significant association with badger age. The alpha fraction was however significantly associated with sex, with the highest concentrations in female badgers (Table 6.3).

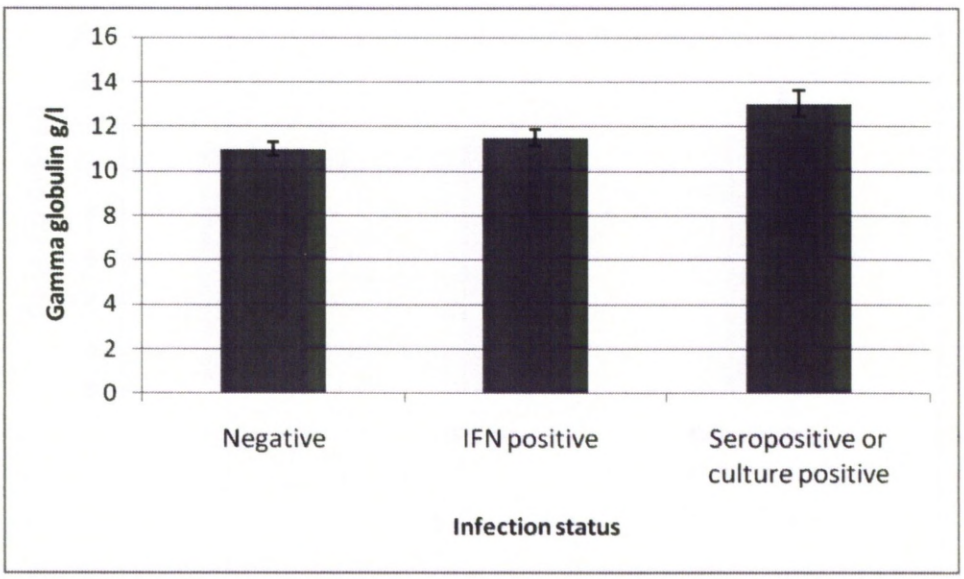

Figure 6.2. Mean serum gamma globulin concentrations from 281 captures of 87 individual badgers of differing infection status from selected social groups (May 2007 to January 2010 inclusive). Error bars represent SEM. Overrall mean = 11.47 g/l.

Table 6.3. Results of GLMMs to identify serum protein correlates of *M. bovis* infection in badgers, whilst controlling for the presence of active bite wounds, season, sex and age, with individual badger fitted as a random effect (n=281, May 2007 to January 2010 inclusive).

Response	Explanatory variables	Test statistic	P value
Albumin g/l	Primary infection status	$F_{(2,181.8)} = 0.18$	0.832
	Active bite wound	$F_{(2,251.7)} = 3.66$	0.057
	Season	$F_{(3,226.5)} = 13.77$	**<0.001**
	Sex	$F_{(1,74.4)} = 0.00$	0.991
	Age	$F_{(1,271.0)} = 8.26$	**0.004**
Total globulin g/l	Primary infection status	$F_{(2,272.0)} = 2.09$	0.126
	Active bite wound	$F_{(2,272.0)} = 1.16$	0.283
	Season	$F_{(2,272.0)} = 21.07$	**<0.001**
	Sex	$F_{(2,272.0)} = 1.56$	0.213
	Age	$F_{(2,272.0)} = 45.20$	**<0.001**
Alpha g/l	Primary infection status	$F_{(2,98.0)} = 0.11$	0.899
	Active bite wound	$F_{(2,271.0)} = 1.93$	0.166
	Season	$F_{(3,251.4)} = 12.31$	**<0.001**
	Sex	$F_{(1,60.5)} = 9.90$	**0.003**
	Age	$F_{(1,253.0)} = 1.34$	0.248
Beta g/l	Primary infection status	$F_{(2,82.0)} = 0.76$	0.472
	Active bite wound	$F_{(1,270.8)} = 0.15$	0.697
	Season	$F_{(3,256.0)} = 21.97$	**<0.001**
	Sex	$F_{(1,54.1)} = 0.04$	0.847
	Age	$F_{(1,244.4)} = 66.36$	**<0.001**
Gamma g/l	Primary infection status	$F_{(2,134.3)} = 3.21$	**0.043***
	***Predicted mean values: Negative = 11.67g/l; IFN = 11.64 g/l; Seropositive/excretor = 13.33 g/l**		
	Active bite wound	$F_{(1,262.5)} = 4.57$	**0.033**
	Season	$F_{(3,234.4)} = 3.34$	**0.020**
	Sex	$F_{(1,63.5)} = 1.79$	0.185
	Age	$F_{(1,269.7)} = 22.44$	**<0.001**

There were no significant associations between infection status and the remaining blood biochemical parameters (Table 6.4). There were some significant associations with season, such that creatinine concentrations were highest in the winter months, and GGT and bilirubin were highest in the summer months. Age was a significant factor for several parameters, with higher concentrations of GGT, calcium and bilirubin in cubs, and lower concentrations of creatinine in cubs. The concentrations of GGT and bilirubin were significantly higher in female badgers than males.

Table 6.4. Results of GLMMs to identify blood biochemistry correlates of *M. bovis* infection in badgers, whilst controlling for season, sex and age, with individual badger fitted as a random effect (n=281, May 2007 to January 2010 inclusive).

Response	**Explanatory variables**	**Test statistic**	**P value**
GGT U/l	Primary infection status	$F_{(2,96.6)} = 1.20$	0.306
	Season	$F_{(3,259.3)} = 20.40$	**<0.001**
	Sex	$F_{(1,65.2)} = 6.63$	**0.012**
	Age	$F_{(1,247.7)} = 10.70$	**0.001**
Creatinine mmol/l	Primary infection status	$F_{(2,116.2)} = 2.65$	0.075
	Season	$F_{(3,249.9)} = 26.27$	**<0.001**
	Sex	$F_{(1,67.2)} = 0.04$	0.841
	Age	$F_{(1,259.2)} = 9.41$	**0.002**
Calcium mmol/l	Primary infection status	$F_{(2,273.0)} = 0.75$	0.473
	Season	$F_{(2,273.0)} = 2.13$	0.097
	Sex	$F_{(2,273.0)} = 3.04$	0.082
	Age	$F_{(2,273.0)} = 40.37$	**<0.001**
Bilirubin µmol/l	Primary infection status	$F_{(2,169.6)} = 1.16$	0.317
	Season	$F_{(3,224.9)} = 7.63$	**<0.001**
	Sex	$F_{(1,69.0)} = 5.65$	**0.020**
	Age	$F_{(1,272.9)} = 6.07$	**0.014**

6.3.2 Parasitological correlates of *M. bovis* infection

At least two distinct helminth ova of typical strongyle morphology (Figure 6.3) were recorded from microscopic examination using faecal egg flotation. Based on previous reports, the likely species are *Molineus patens* and *Uncinaria stenocephala.* In addition, ova with morphological features typical of *Capillaria* spp. (Figure 6.4) were recorded, the most likely species being *Capillaria erinacei* (Hancox 1980, Jones *et al.* 1980). Two distinct species of coccidia were also recorded, and identified as *Eimeria melis* (Figure 6.5) (Kotlan & Pospech 1933) and *Isospora melis* (Pellérdy 1955) respectively (Figure 6.6). In addition several first stage nematode larvae were seen, but these were often degenerate. Based on previous reports, the larvae were likely to be *Aelurostrongylus falciformis* larvae (Hancox 1980, Jones *et al.* 1980), but no attempt was made to quantify their presence or confirm their identification in the present study and they are not included in any analyses.

Figure 6.3. Strongyle ovum possibly *Uncinaria stenocephala* or *Molineus patens*, observed in badger faecal samples.

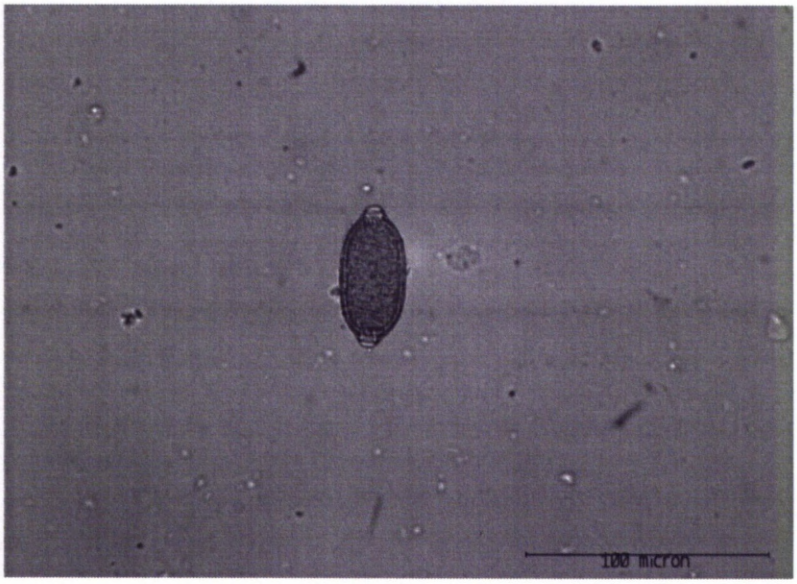

Figure 6.4. Capillariid ovum showing typical bipolar operculum (possibly *Capillaria erinacei),* observed in badger faecal samples.

Figure 6.5. *Eimeria melis* oocysts, sporulated with four sporocysts, observed in badger faecal samples.

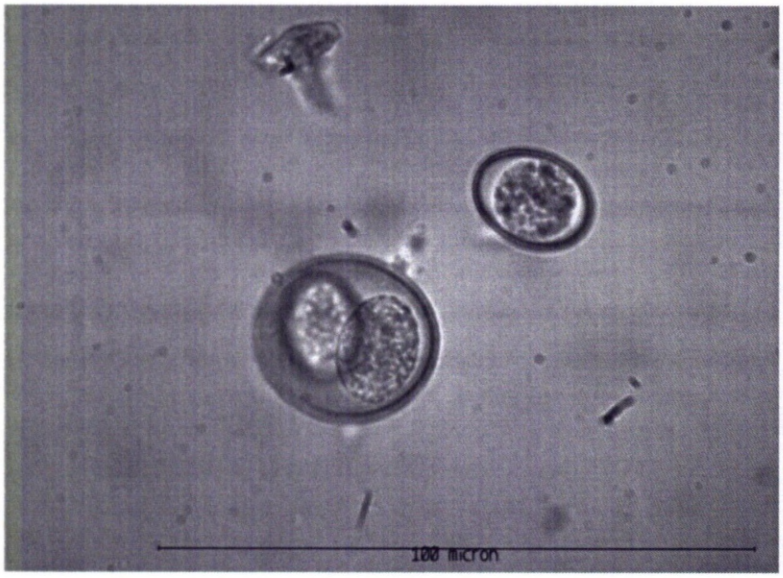

Figure 6.6. *Isospora melis* oocyst (centre), sporulated with two sporocysts, and a smaller, as yet unsporulated, *Eimeria melis* oocyst (right), observed in badger faecal samples.

Nematode ova were present in 55% of faecal samples from cubs and in 63% from adults. *Eimeria melis* and *Isospora melis* oocysts were detected in 73% and 45% of faecal samples from cubs respectively, in contrast to only 22% and 9% of adults (Figure 6.7).

The mean intensity of infection for each parasite was higher in cubs than adults (Figure 6.8), but of particular note were the heavy burdens of *Eimeria melis*, with a mean intensity in cubs of over 60 000 oocysts per gram of faeces (Figure 6.8).

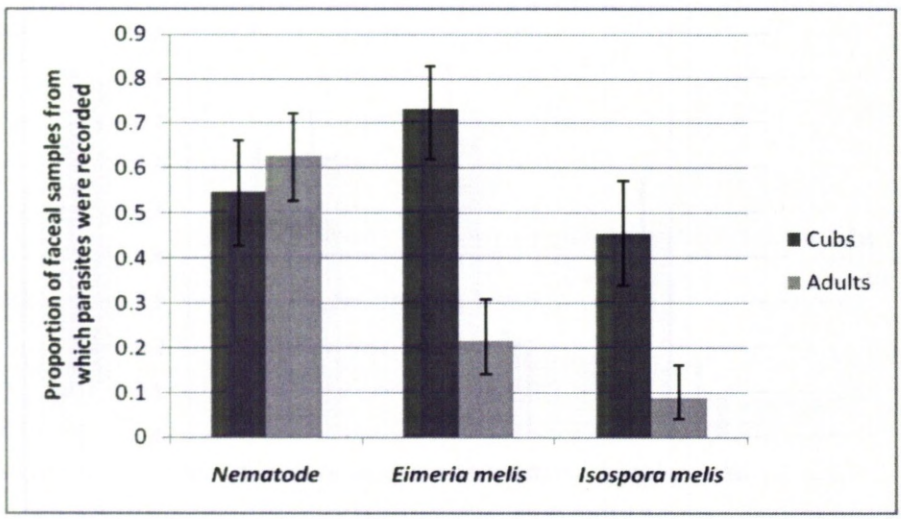

Figure 6.7. Prevalence of nematodes and coccidia in 177 badger faecal samples from 75 badgers (May 2007 and January 2010 inclusive). Error bars represent 95% confidence intervals.

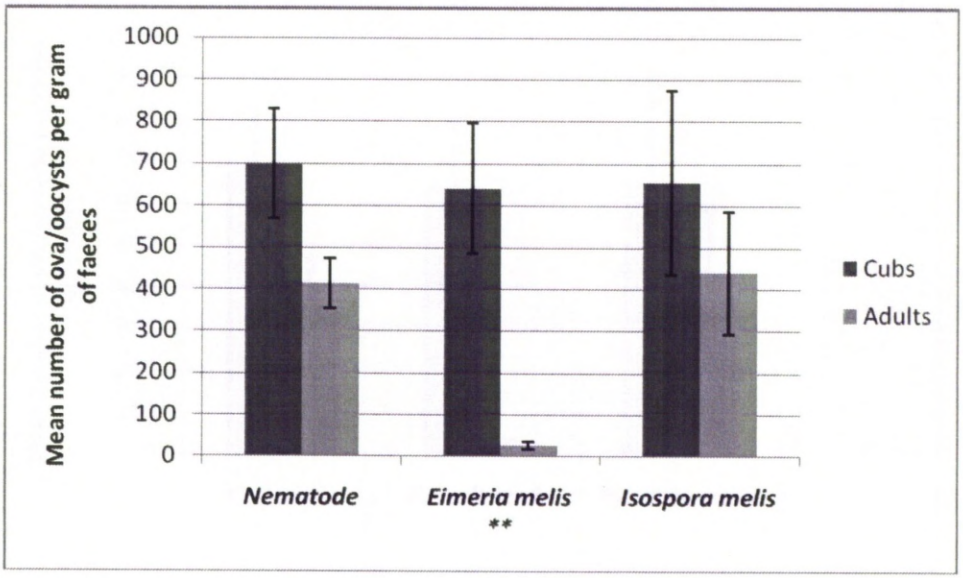

Figure 6.8. Mean intensity of infection for nematodes and coccida in 177 badger faecal samples from 75 badgers (May 2007 to January 2010). Error bars represent SEM.

** *Eimeria melis* oocyst counts were divided by 100.

There were no significant associations between either the presence or intensity of any of the parasite species and the magnitude of the IFN response amongst captures of badgers with current or previous evidence of infection with *M. bovis* (Tables 6.5 and 6.6). However, the likelihood of detection of both species of coccidia was significantly higher in cubs than adults ($p<0.001$) (Figure 6.7, Table 6.5), but there was no significant difference in the likelihood of detection of nematode ova between cubs and adults. In addition, the intensity of infection with *Eimeria melis* was significantly higher in cubs than in adults ($p<0.001$) (Figure 6.8, Table 6.6). However, there was no significant difference in the intensities of nematode infections between cubs and adults. Low numbers of samples positive for *Isospora melis* prevented statistical analysis of the intensity of infection.

Table 6.5. Associations between the presence of nematode ova, *Eimeria melis* and *Isospora melis* oocysts respectively, and the magnitude of the IFN response (OD value B-A, log transformed) in infected badgers. Analyses controlled for the effects of age (cub or adult), season, sex, and repeat captures of the same individual (n=177, May 2007 to January 2010 inclusive). Individual badger was fitted as a random effect in all models.

Response variable – presence or absence	**Explanatory variable**	**Test statistic**	**P value**
Nematode ova	IFN response	$\chi^2_{(1)} = 1.76$	0.184
	Age	$\chi^2_{(1)} = 0.72$	0.397
	Season	$\chi^2_{(3)} = 2.77$	**0.040**
	Sex	$\chi^2_{(1)} = 2.00$	0.157
Eimeria melis	IFN response	$\chi^2_{(1)} = 1.57$	0.210
	Age	$\chi^2_{(1)} = 19.86$ (C>A)	**<0.001**
	Season	$\chi^2_{(3)} = 1.62$	0.182
	Sex	$\chi^2_{(1)} = 0.42$	0.517
Isospora melis	IFN magnitude	$\chi^2_{(1)} = 1.14$	0.286
	Age	$\chi^2_{(1)} = 17.63$ (C>A)	**<0.001**
	Season	$\chi^2_{(3)} = 2.01$	0.110
	Sex	$\chi^2_{(1)} = 1.14$	0.286

Table 6.6. Associations between the intensity of nematode ova and *Eimeria melis* oocysts respectively, and the magnitude of the IFN response (OD value B-A, log transformed) in infected badgers. Analyses controlled for the effects of age (cub or adult), season, sex, and repeat captures of the same individual (n=177, May 2007 to January 2010 inclusive). Individual badger was fitted as a random effect in both models.

Response variable - intensity of infection (log transformed)	**Explanatory variable**	**Test statistic**	**P value**
Nematode ova	IFN response	$F_{(1,45.0)} = 0.10$	0.751
	Age	$F_{(1,35.3)} = 1.09$	0.303
	Season	$F_{(3,35.8)} = 0.68$	0.569
	Sex	$F_{(1,19.7)} = 0.05$	0.831
Eimeria melis	IFN response	$F_{(1,28.2)} = 0.15$	0.706
	Age	$F_{(1,31.3)} = 14.13$	**<0.001**
	Season	$F_{(3,26.4)} = 4.26$	**0.014**
	Sex	$F_{(1,23.6)} = 0.12$	0.735

6.3.3 Parasite burdens and badger body condition

The body condition of badger cubs was significantly negatively associated with the presence of nematode infections (p=0.002) (Figure 6.9, Table 6.7). There were however, no significant associations between the presence of either species of coccidia and body condition. There were no significant associations between the intensity of parasite infections and body condition in cubs (Table 6.8). For adult badgers there were no significant associations between body condition and the presence or intensity of any of the parasites detected by faecal flotation (Tables 6.7 and 6.8).

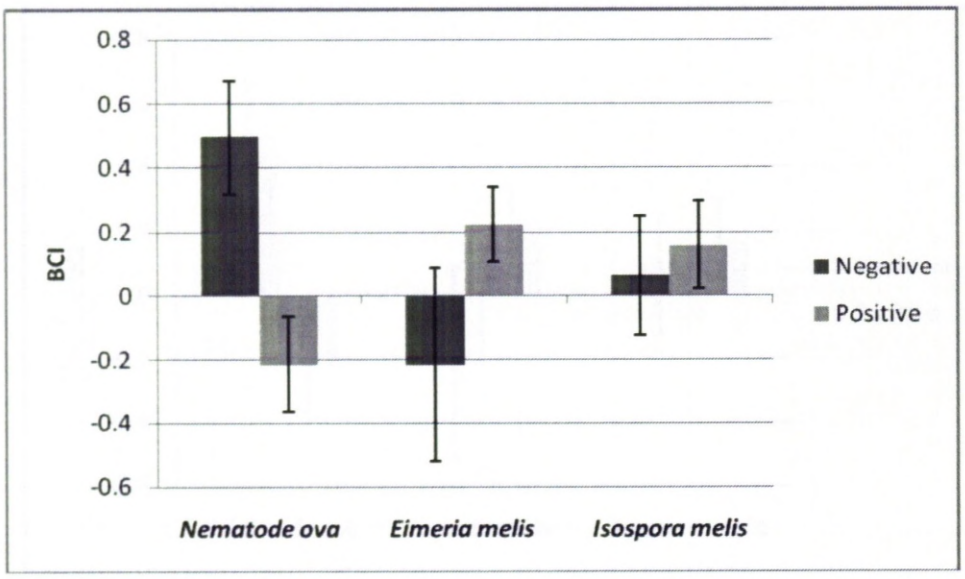

Figure 6.9. Body condition indices for badger cubs in association with the presence or absence of parasites detected on faecal examination. Error bars represent SEM.

Table 6.7. Associations between body condition and the occurrence of ova/oocysts in faecal samples from badgers (May 2007 to January 2010 inclusive). Individual badger was fitted as a random effect in both models.

Response variable	**Explanatory variable Presence or absence**	**Test statistic**	**P value**
Cub BCI N=75	Nematodes	$F_{(1,68.2)} = 10.44$	**0.002**
	Eimeria melis	$F_{(1,63.3)} = 3.63$	0.061
	Isospora melis	$F_{(1,68.4)} = 0.11$	0.741
	Sex	$F_{(1,43.2)} = 0.00$	0.997
Adult BCI N=82	Nematodes	$F_{(1,94.9)} = 2.41$	0.124
	Eimeria melis	$F_{(1,94.5)} = 0.94$	0.336
	Isospora melis	$F_{(1,79.5)} = 2.46$	0.121
	Sex	$F_{(1,35.1)} = 1.68$	0.203
	Excretion status	$F_{(1,45.8)} = 0.03$	0.873

Table 6.8. Associations between badger body condition and intensity of parasite burdens (log transformed) as estimated from faecal ova/oocyst counts (May 2007 to January 2010 inclusive). Individual badger was fitted as a random effect in both models.

Response variable	**Explanatory variable**	**Test statistic**	**P value**
Cub BCI	Nematode egg count	$F_{(1,12.0)} = 3.33$	0.093
	Eimeria melis oocyst count	$F_{(1,12.0)} = 2.13$	0.170
	Isospora melis oocyst count	$F_{(1,12.0)} = 0.20$	0.666
	Sex	$F_{(1,12.0)} = 1.64$	0.224
Adult BCI	Nematode egg count	$F_{(1,8.0)} = 0.04$	0.841
	Eimeria melis oocyst count	$F_{(1,9.0)} = 0.04$	0.848
	Isospora melis oocyst count	Excluded from model, sample size too small	
	Sex	$F_{(1,7.3)} = 2.33$	0.169
	Excretion status	$F_{(1,7.5)} = 0.41$	0.539

6.4 Discussion

The aim of this chapter was to identify any associations between haematological and blood biochemical parameters, and *M. bovis* infection status, as detected by the diagnostic tests currently available in the live badger. In addition, data were analysed to test for correlations between intestinal endoparasite burdens and the magnitude of the cell-mediated response to *M. bovis* infection.

In the present study there was evidence of an increase in the total white cell count (leucocytosis) in badgers that were seropositive or identified as *M. bovis* excretors, but this was not associated with significant changes in any particular type of white blood cell type (Figure 6.1 and Table 6.2). This is consistent with a generalised leucocytosis with no predominant cell type. In addition, significantly higher concentrations of gamma globulins were observed in badgers with more progressive *M. bovis* infection (as indicated by a seropositive response or bacterial excretion), and in badgers with active bite wounds (Figure 6.2 and Table 6.3). The gamma globulin fraction, as determined in this study using SPE, comprises predominantly immunoglobulins (or antibodies) (Abate *et al.* 2000), but studies in dogs have shown that if C-reactive protein (CRP) is significantly increased, it will also be detected in the gamma region (Yamamoto *et al.* 1992). CRP in badgers has not been characterised, nor has its role as an acute phase protein been evaluated. Without specific protein assays it is not possible to determine what proportion of the observed increase in gamma globulins was due to CRP, or whether it was solely due to the antibody response in association with both *M. bovis* infection and active bite wounds. Badger age, sex and season were controlled for in the analyses, and age associations were generally consistent with those found in other studies, with lower red cell counts, lower concentrations of haemoglobin, serum proteins and creatinine, and higher concentrations of calcium in cubs (Winnacker *et al.* 2008). Concentrations of alpha proteins were significantly higher in females than males, and seasonal associations were significant for all the serum proteins, consistent with other findings (Domingo-Roura *et al.* 2001). Further interpretation is outwith these analyses and would be necessarily speculative without specific protein assays and consideration of the likely complexity of interactions between age, season and sex in association with reproductive, nutritional and social stress. However, serum proteins may be worthy of closer investigation with respect to understanding more about sex and age related differences in responses to physiological stressors at different times of the year, which in turn may underpin explanations for age and sex related differences in infection acquisition and disease progression.

Although the total white blood cell counts were significantly higher in badgers with evidence of seroconversion or *M. bovis* excretion (Figure 6.1 and Table 6.2), this finding should be interpreted with caution. The magnitude of the increase appears to be modest in comparison to reported normal reference ranges (circa 5-14 x 10^9/l) for domestic dogs and cats (Kerr 1989), and the 95% CI for the mean value of 7.28 – 8.44 x10^9/l for wild badgers, reported in Winnacker *et al.* (2008). The gamma globulin concentrations were also significantly higher in badgers with evidence of seroconversion or *M. bovis* excretion (Figure 6.2 and Table 6.3). However, leucocytosis and hypergammaglobulinaemia are unlikely to be of any specific value as diagnostic or prognostic indicators with respect to *M. bovis* infection, given the frequency of occurrence of bite wounds (Delahay *et al.* 2006a), which could cause similar changes.

There was no evidence of any increase in APPs that migrate to the alpha and beta regions, although specific protein assays would be a more sensitive method for their detection, and analyses of other blood biochemical measures found no significant associations with *M. bovis* infection (Tables 6.3 and 6.4). It is difficult to make direct comparisons with other studies since the methods of classifying infection status vary widely, from using the culture of tissue samples collected *post-mortem* (Chambers *et al.* 2000) through to experimental infection models where the time and route of infection and the infective dose were all controlled (Mahmood *et al.* 1988). The lack of any evidence of changes in clinical pathology parameters is, however, consistent with minimal physiological impact of *M. bovis* infection for the majority of badgers captured during this study period.

Due to the importance of tuberculosis in the human population, particularly in the developing world, there has been interest in the role of helminth infections in modulating immune responses both to infection (Bentwich *et al.* 1999, Elias *et al.* 2001, Resende Co *et al.* 2006,) and to vaccination (van Riet *et al.* 2007, Elias *et al.* 2008). However, the role of co-infections in the epidemiology of tuberculosis in wildlife systems has not been extensively studied. Wild badgers are infected with a range of pathogens, and interactions may play a significant part in the pathogenesis of *M. bovis* infection. One aim of the present study was to investigate associations between the magnitude of the cell-mediated immune response to *M. bovis* infection in individual badgers, and helminth and coccidian infections. The parasites observed in the present study were consistent with those reported from previous studies on badgers in the UK (Hancox 1980, Jones *et al.* 1980, Anwar *et al.* 2000, Dale 2005), with three common nematode species and two common coccidian species as described. There were, however, no significant associations between any of the parasites

detected on faecal egg flotation, and the magnitude of the cell-mediated response to *M. bovis* infection (Tables 6.5 and 6.6). These findings are in contrast to a reduced IFN response observed in cattle infected with *Fasciola hepatica* (Flynn *et al.* 2009). It may be that the approach used was not sensitive enough to detect associations, or that badgers are very well-adapted to the intestinal pathogens assessed in this study, and they have little or no impact on immune responses and susceptibility to *M. bovis* infection.

There were however, significant age associations for the presence and intensity of coccidian infections. The prevalence and intensity of infection with *Eimeria melis* were greater in cubs than adults (Figures 6.7 and 6.8 and Tables 6.5 and 6.6). *Isospora melis* was more prevalent in cubs, but intensity of infection did not vary with age. Nematode infections were also more common in cubs, but this relationship was non-significant. These findings are broadly consistent with parasitic infections in domesticated species in which immunity to coccidial infections develops more rapidly over time than immunity to helminth infections (Taylor *et al.* 2007).

The present study found no direct associations between parasite burdens *and M. bovis* infection, but the body condition of cubs was significantly negatively associated with the presence of nematode infections (Table 6.7). There was however, no significant association between the intensity of nematode infections and cub body condition (Table 6.8). These findings could be consistent either with nematode infections having a negative impact on body condition in badger cubs, or with cubs in poor condition being more susceptible to nematode infection. The latter may be more likely as there was no significant association between the intensity of the nematode infections and cub body condition.

Interestingly, there was no significant association between cub body condition and both coccidian species (Tables 6.7 and 6.8). This is surprising in the light of a mean intensity of infection for *Eimeria melis* of over 60 000 oocysts per gram of faeces (Figure 6.8). These results are consistent with adequate host adaptation to infection with coccidia or low parasite pathogenicity, and support the hypothesis of age acquired immunity rather than infection associated mortality, although this was not specifically tested in these analyses. With respect to infection with *Eimeria melis* in cubs, this interpretation does conflict with previous conclusions from an Oxfordshire population where impaired cub growth and increased cub mortality were associated with coccidiosis in cubs (Newman *et al.* 2001). Impaired cub growth was however, based on measurements taken the following year, in contrast to the present study which used concurrent measures of coccidial infection and body condition. There were no significant associations between adult body condition and

any of the parasitic infections, again suggesting adequate host adaptation in adults to infection with both coccidia and nematodes, but any potential for annual variation would be better controlled for with observations over a longer time period.

In conclusion, this study found very little correlation between *M. bovis* infection and haematological and blood biochemical parameters. The only significant observations were either non-specific and/or small in magnitude. This suggests that for the majority of infected badgers sampled during this study period, *M. bovis* infection had minimal physiological impact. In addition, there were no significant associations between intestinal parasite burdens and the magnitude of the cell-mediated response to *M. bovis* infection, consistent with low levels of interaction between the pathogens studied.

CHAPTER SEVEN: General Discussion

7.1 Introduction

The aims of the present study were to identify correlates of the presence and progression of *M. bovis* infection in individual badgers. To do this required an understanding of the pathogenesis of infection in badgers and how this was correlated with currently available diagnostic tests, whilst recognising limitations in test performance.

The impact of infection on individual badgers may change their behaviour, survival and reproductive success, all potentially affecting the likelihood of onward transmission within the social group and more widely to other social groups and potentially other species, particularly livestock. Understanding these effects is important for the development and optimisation of potential disease control strategies.

The Woodchester Park badger capture dataset represents one of the longest running longitudinal studies of a free-living wild mammal. Trapping effort has been largely consistent over the periods used in the analyses presented here, and although capture probabilities vary annually (ranging from 0.61 in 2004 to 0.85 in 1998) they remain high (Fera 2011). As previously discussed (General Methods), it is probable that the routine trapping captures most of the resident population, ensuring that the data are indeed representative of the whole population. At an individual badger level, differing numbers of capture events and intervals of varying duration between captures complicate analysis of the dataset. Mixed model approaches using fixed and random effects to partition variation in response variables have been used in previous research and in this thesis, but more advanced Bayesian methodologies may enhance analysis of this valuable dataset (Fera 2011).

The social structure of the study population and the assignment of individuals to a social group at each capture event gives the dataset an inherently hierarchical structure. Indeed many of the analyses in the thesis have used the infection status of certain individuals within the social group as a proxy for infection risk to others in the group, particularly cubs.

It is also possible that the social group could contribute directly to variations in body condition owing to genetic relatedness of individuals and inherent differences in the characteristics of group territories. For example, some territories may contain habitat of

relatively higher foraging quality than others (see Delahay *et al.* 2006b). In the models to partition the variation in body condition, this approach was not used, and variation in body condition in association with badger social group was not taken into account. The potential effect of this might be to over-estimate the variation associated with the other explanatory factors in the model. However, the likelihood of such bias may be small as the effects of social group territory on condition of the residents are likely to vary in time as a result of changes in land use, seasonal and climatic factors, and may not be uniform at an individual level within the group due to differential habitat use by badgers of differing age and sex (see Delahay *et al.* 2006b).

7.2 Diagnostic tests as cornerstones for monitoring infection

Diagnostic testing for tuberculous mycobacterial infections is complicated by a highly variable interaction between the immune system of the host and the pathogen. Historically, tests have been limited to serological assays, and mycobacterial culture of samples collected from live animals as in this study, or the culture of tissues taken *post-mortem*. More recently, tests for cell-mediated immune responses to *M. bovis* in the badger, in particular the gamma interferon assay, have been developed, validated and made available for research purposes (Corner *et al.* 2011).

The considerable limitations of each of the diagnostic tests have already been discussed, and it is accepted that infection prevalence in this study population is consequently underestimated. However, using a combination of diagnostic test results at each capture event, and interpreting a suite of results based on the model of progression from cell-mediated responses to serological responses to excretion, it is possible to assign infection status at each capture event. In addition, despite the limitations of the tests, their performance has generally been consistent in the Woodchester Park badger population. The only exception was the Brock ELISA in recent years, results of which have not been used in these analyses. Consistency of test results is supported by considerable levels of both spatial and temporal correlation in the detection of infection observed in the Woodchester Park population (Cheeseman *et al.* 1988, Delahay *et al.* 2000a). Furthermore, the majority of analyses using diagnostic tests in the present study were based on a comparison *between* badgers, rather than absolute prevalence data, and where test limitations did not apply equally, for example in relation to the differences between cubs and adults, this was taken into consideration. Where absolute prevalence data were used, test limitations were taken into consideration in their interpretation.

Mycobacterial culture from samples taken from the live badger is considered to be an insensitive method for the diagnosis of the presence of *M. bovis* infection (Pritchard *et al.* 1986). The mycobacterial culture of tissue samples taken *post-mortem* is considered the gold-standard against which all other tests are validated, but there is good evidence to show that the intensity of the *post-mortem* examination and associated sample collection affect the sensitivity of this approach (Crawshaw *et al.* 2008, Murphy *et al.* 2010). Mycobacterial culture however, has a high specificity of close to 100% (Pritchard *et al.* 1986, Clifton-Hadley *et al.* 1993).

Despite the low sensitivity of culture, the analyses reported in the present study showed that the detection of *M. bovis* in badgers at live capture was correlated with loss of condition (Chapter Three). Hence it appears that positive mycobacterial culture is a more sensitive detector of the progression of infection to the point at which badgers are physiologically compromised, even if it is an insensitive detector of the presence of infection. This may be of value in the context of assessing the welfare implications of *M. bovis* infection both at the individual and population level. In addition, the presence of breeding females excreting *M. bovis* was significantly associated with the likelihood of cub infection acquisition and progression (Chapters Four and Five). This is consistent with the detection of excretion status being related to infectiousness, but the close proximity of susceptible cubs to a breeding female may also be a factor.

The earliest serological test to be developed, validated and used routinely in the research programmes for bovine tuberculosis in the badger was the Brock ELISA (Goodger *et al.* 1994), and results from this test were used in the retrospective analyses in Chapters Three and Four. The test has a low sensitivity, but a higher specificity and more importantly its sensitivity increases with progressive infection, probably in direct association with an increasing *M. bovis* antigenic load. More recently, the Stat-Pak assay has become available (Chambers *et al.* 2008), and was used in analyses in Chapters Five and Six. Its main advantage is not in enhanced test properties, but in ease of use and its potential value as a field test. Sensitivity and specificity are similar to the Brock ELISA.

The IFN assay is more sensitive than the serological assays. However, test sensitivity does appear to be lower in cubs (Chambers *et al.* 2009), a factor which was investigated and taken into account by appropriate modification of results in the analyses in Chapter Five of the present study. Results presented here revealed that there was some potential predictive value of the magnitude of the IFN response early in the infection process, with higher levels correlated with a greater likelihood of progression. The underlying mechanism

for this correlation could be associated with the route of infection, infectious dose or differences between individual responses to *M. bovis* infection, or a combination of all three. Further understanding of the specific mechanism involved may be largely of academic interest, but from a more practical perspective, it may be that there is true predictive value in the magnitude of the IFN response at or close to the incident event. Whether this can really be utilised in any potential control strategy appears unlikely at this stage, due to the confidence limits associated with defining a cut-off point, and perhaps more importantly, the limitations of a test that requires a specialist laboratory, prompt sample handling and a lengthy test procedure. In addition, analyses supported the hypothesis that cell-mediated responses to *M. bovis* fluctuate following infection and generally fade with time (Figure 5.6), even in badgers with evidence of progressive infection. In such badgers, this could be consistent with exhaustion of the IFN response or anergy. In badgers with no evidence of progressive infection, this could be considered consistent with latency, with peaks in IFN responses in association with periods of mycobacterial multiplication. The results presented here are the first longitudinal measures of the cell-mediated response in free-living wild badgers naturally infected with *M. bovis* and contribute significantly therefore, in conjunction with studies of experimentally infected badgers, to our understanding of the immune responses of the badger to *M. bovis* infection.

The correlation of life-history traits reported in the present study required categorisation of badgers into epidemiologically meaningful groups. This was based on a model in which early infection was characterised by a cell-mediated response, as detected by the IFN test. The progression of infection in an individual was characterised by detection of a serological response, and ultimately detection of *M. bovis* excretion. In badgers with evidence of an IFN response, but no concurrent or subsequent evidence of a serological response or *M. bovis* excretion, this was interpreted as control or possibly even clearance of infection. This approach relied on longitudinal data from the re-capture of individuals, which has the advantage of increasing the sensitivity of detection of responses to infection with *M. bovis*.

Interpretation of diagnostic test results remains a significant challenge, particularly in wildlife species, where there may be no widely accepted gold-standard diagnostic test. The application of Bayesian statistics, using continuous data to describe infection on a probabilistic scale may be helpful in the future. An early example of this approach, applied to the diagnosis of *M. bovis* in badgers is described in Drewe *et al.* (2010).

7.3 Physiological effects at an individual level

By definition, pathogenic infections cause disease through effects on physiological homeostasis. Detecting these effects in direct association with a specific infectious agent is often problematic and unrewarding, particularly for chronic infections which tend to be insidious in onset, with slow clinical progression and non-specific clinical signs. However, using measures indicative of disturbances to physiological homeostasis in individual badgers, such as body condition, survival times, reproductive success and clinical pathology markers, significant effects associated with infection can help us better understand the dynamics of disease at the social group and population level.

The pathogenesis of *M. bovis* infection in free-living wildlife is complicated by the interaction with extrinsic and intrinsic stressors such as food availability, reproductive stress, environmental stress and social stress, and their variable effects in animals of different sex or age. Indeed it has been hypothesised that these types of stressors may be particularly important with respect to the triggering of mycobacterial excretion in infected badgers (Gallagher & Clifton-Hadley 2000). One of the aims of the present study was to tease out the nature of associations between certain stressors and infection progression in badgers using data from the long-term Woodchester Park trapping and sampling project.

Analyses in Chapter Six revealed few significant associations between any of the clinical pathology parameters and *M. bovis* infection, with the exception of an increase in gamma globulins in association with seropositive or excretion status. As discussed, without more specific protein assays, it is not possible to determine whether this was due to antibody responses or in part to an increase in C-reactive protein, an acute phase protein that migrates to the gamma band during serum protein electrophoresis. In addition, the magnitude of the cell-mediated response to *M. bovis* infection was not correlated with intestinal endoparasite burdens. The lack of any significant findings is consistent with a species that is very well adapted to infection with its endemic pathogens, including *M. bovis*. One finding of interest was the negative association between cub body condition and the presence of nematode infections. Since there was no significant association between the intensity of nematode infections and body condition however, it would appear more likely that the direction of the association is such that poor condition increases susceptibility to nematode infection. This raises some interesting questions about the interaction between cub susceptibility to infection and body condition which may be worthy of more specific consideration in future work.

The analyses in Chapter Three of the present study aimed to specifically investigate associations between body condition and infection status and how these varied with season and sex. The results broadly indicated significant negative correlation between badger body condition and positive *M. bovis* excretion status, but no association between body condition and seropositivity. In addition, there was no evidence to support a loss of body condition triggering the onset of excretion of bacteria. This is consistent with the seasonal cyclicity of body weight being well tolerated by badgers, and is supported by other studies suggesting that there are underlying intrinsic, possibly hormonal mechanisms for seasonal weight fluctuation, independent of food availability (Maurel & Boissin 1979, Kruuk & Parish 1983). Nevertheless when food resources are severely restricted, for example in years of very dry spring and summer weather, there may be associated physiological stress, potentially triggering the onset of bacterial excretion.

The negative association between excretion and body condition illustrates that only when infection has progressed to the point of detectable excretion, does body condition appear to be affected. Interestingly, however, when the data were broken down by sex and season, the negative association between excretion status and body condition during the summer was still significant for both sexes, but during the autumn and winter, the body condition of females excreting *M. bovis* was indistinguishable from that of non-excretors, even if they had also shown evidence of breeding that year. It would appear that, regardless of the specific mechanism underlying the cyclicity of body condition (nutritional restriction or intrinsic adaptation), excretor badgers of both sexes were unable to maintain body condition during the summer months. During this period breeding females were recovering from the additional physiological drain of lactation. What was perhaps more surprising was the ability of females to regain weight into the autumn and winter, despite their excretion status, such that they were indistinguishable from non-excretor status badgers. This resilience amongst breeding females is of particular relevance for reproductive output of the population, since successful breeding in the following year has been positively associated with body condition in the previous winter (Woodroffe & Macdonald 1995, Delahay *et al.* 2006b).

There were further sex associated differences in the survival times from the point of detection of excretion to an individual's last capture, consistent with female badgers surviving longer than males. It would therefore appear that the direct effects of *M. bovis* infection differ between the sexes, with females showing a greater resilience to condition loss and lower rates of mortality subsequent to the onset of excretion. These differences

are consistent with more vigorous cell-mediated responses in females (Boumann *et al.* 2005), although IFN responses did not differ in the present study (Chapter Five).

With respect to reproductive success, there was no evidence in any of the analyses of a significant negative effect of *M. bovis* infection on female reproductive success. The likelihood of detecting reproductive activity in female badgers was not significantly associated with infection status, and the number of cubs reared within a social group was not significantly associated with the infection status of the resident breeding adult female badgers. Furthermore, females that were excreting *M. bovis* were detected as reproductively active up to eight years from the point of detection of excretion, in line with the long survival times discussed earlier. This is in contrast to observations of depressed fecundity in female African buffalo (*Syncerus caffer*) infected with *M. bovis* (Jolles *et al.* 2005).

Pathogens can influence host population dynamics through disease-induced effects on both individual survival and fecundity (Anderson & May 1978). The findings in the present study suggest that the resilience of breeding female badgers to the negative effects of *M. bovis* infection may limit the effects of the pathogen on population dynamics. However, this relationship may only hold for badger populations in similar habitat, of similar density, and with similar pathogen prevalence.

At a population level, the contribution of individuals to both the maintenance and spread of infection are a function of both the duration of infectiousness and the severity of infection or the numbers of bacilli excreted. This is in combination with the duration and frequency of contacts between individuals. The sex related differences observed in the present study suggest that the duration of infectiousness will be longest in female badgers, and that they play an important role in maintenance of infection within the social group via pseudo-vertical transmission to their cubs. The lower mortality rates of females excreting *M. bovis* combined with their reproductive success ensures that each year cubs are born into an environment where they will be in close proximity to an infectious adult.

Other analyses in Chapter Three investigated whether there was any detectable difference in survival times associated with the clinical presentation of excretion. Consistent with previous research (Gallagher & Nelson 1979, Clifton-Hadley *et al.* 1993), shorter survival times were significantly associated with *M. bovis* positive bite wounds being present at the excretion incident event. In experimental infection models, direct inoculation of *M. bovis* resulted in more aggressive infection (Pritchard *et al.* 1987), and the findings reported here are consistent with bite wounding as a route of infection. With

respect to the epidemiological contribution of bite wounding as a mode of transmission, it should be noted that the relative distribution by age and sex within the population of *M. bovis* positive bite wounds may differ from the relative distribution of all bite wounds. Hence although bite wounding per se is more commonly seen in adults than cubs, and in males more than females (Delahay *et al.* 2006a), the same may not be true for *M. bovis* positive bite wounds. Further work using data on bite wounding and behavioural and social network analyses may provide more specific information on the true contribution of bite wounding to the epidemiology of bovine tuberculosis in badgers.

In addition to positive bite wounds, the survival times of badgers presenting with *M. bovis* positive lymph node abscesses at the excretion incident event were significantly shorter than those without (test statistics indicated that the shorter survival was roughly equivalent for bite wounds and lymph node abscesses). It is debatable whether this presentation represents a truly distinct route of infection (Murphy *et al.* 2010), or whether it is merely an extension of progressive respiratory infection (Corner *et al.* 2011), but it does appear to be a poor prognostic indicator.

The contribution to the maintenance and spread of *M. bovis* within the population of badgers with positive bite wounds or lymph node abscesses may be of shorter duration than those without. However, the aggressive nature of the ensuing infection may represent a more debilitating infection with higher numbers of bacilli being excreted at increased frequency, and a greater negative welfare impact for the individual badger.

7.4 Badger welfare in association with *M. bovis* infection

Whether human beings should be concerned about wild animal welfare, and in particular whether we should intervene, are complex ethical questions and opinions differ widely (Kirkwood *et al.* 1994). In general, if there is reason to believe human activity is directly responsible for negative welfare effects, there is a greater acceptance for responsibility and intervention, such as the rescue and rehabilitation of sea-birds following oil spills for example (Newman *et al.* 2000). In the case of *M. bovis* infection in badgers, opinions are divided as to whether intervention on welfare grounds is either necessary or justified. Welfare concerns for individual badgers infected with *M. bovis* have been cited as a valid reason for culling interventions by some (Gallagher *et al.* 2006), but badgers are infected with many pathogens, none of which seem to trigger this level of debate.

Putting aside the ethical issues relating to intervention, whether bovine tuberculosis is a significant negative welfare issue can be addressed in a more objective manner, by considering the levels of pain and stress the disease causes. Stress is a difficult concept, but

can be deemed to have a negative welfare impact if the level of stress is such that normal physiological functions, such as immune competence, reproductive function and growth are compromised through diversion of resources (Kirkwood *et al.* 1994). Where disease is determined to be a cause of death, it is clear that normal physiological functions have been severely compromised and the welfare impact will be related to the duration of that compromise. It should also be noted however, that badgers are infected with a wide range of other pathogens, which may all have negative welfare impacts and in addition, experience many natural events that could be deemed to compromise welfare, for example, intra-specific aggression and competition for food resources. The analyses reported here revealed little evidence of physiological compromise until infection had progressed to the point of excretion, and even then, the reproductive success of adult female badgers did not appear be significantly adversely affected, and their ability to increase body weight in the autumn as per the normal seasonal cycle, appeared to be maintained.

Clearly where infection was terminal and condition loss was observed, this would represent a period of physiological compromise and negative welfare. The duration of this period appeared to be shorter in male badgers than females, and in association with *M. bovis* positive bite wounds or lymph node abscesses. However, this short duration may be counter-balanced by greater severity in association with aggressive and severe infection.

These analyses suggest that there are negative welfare issues associated with terminal bovine tuberculosis in badgers, however their severity should be judged in the light of the many other factors that have the potential to affect welfare in a free-living wild animal.

7.5 Social interactions in the epidemiology of bovine tuberculosis in badgers

Following on from the findings of the third chapter, the role of pseudo-vertical transmission was explored further by examining the outcome for cubs in relation to the infection status of breeding females and other adult badgers in their social group of origin. The importance of social structure, and in particular contact behaviour amongst individuals is a relatively new concept in infectious disease epidemiology (Keeling 1999, Cross *et al.* 2009), and has been sparsely applied to wildlife disease models. Studies of bovine tuberculosis infection in meerkats (*Suricata suricatta*) in the Kalahari revealed that being groomed and/or exhibiting high levels of social interaction were not risk factors for the acquisition of infection, but grooming others was a particularly risky behaviour (Drewe 2010). Behavioural studies of badgers, a largely nocturnal species, are intrinsically more difficult than those of diurnally active and easily habituated meerkats (Clutton-Brock *et al.*

1999), but mutual grooming is not an unusual activity in badgers (Neal & Cheeseman 1996). Where tuberculous bite wounds or abscesses are present in a badger, the risk of acquiring *M. bovis* through grooming of that individual would be expected to be high.

The analyses reported here focussed on the contact between cubs and adults within a social group. A strong and significant increased risk of *M. bovis* infection and progression was found in cubs born into social groups with infectious breeding females present, consistent with the close, prolonged levels of contact a dam would have with its offspring. There was a gradient of declining risk associated with the infection status of adults within the social group of origin of cubs from breeding excretor status females to seropositive status breeding females, to excretor status male and non-breeding females, to seropositive male and non-breeding females, to negative status adults respectively. In addition to the acquisition and progression of infection in these cubs, there was a significantly increased risk of cub mortality and no evidence to support any protective role of maternally derived antibody (Chapter Four). These findings are in contrast to a previous study conducted over a short time period and using data from a single social group, which led to the suggestion that transient seropositive responses in cubs (potentially maternally derived) may have a protective role in delaying or even preventing disease progression to excretion (Newell *et al.* 1997).

During both study periods (1982-2005 and 2006-2010), social groups with resident infectious breeding females represented less than 10% of the total number of social groups trapped and sampled. Despite the fact that this is likely to be an underestimate (owing to limitations of test sensitivity as discussed in Chapters Four and Five), it would still appear that these groups are a minority. Nevertheless, infectious breeding females clearly represent a significant risk to cubs within their social groups, and are likely to be important in the maintenance of infection within the population.

Putting all these findings in context in trying to tease out the relative contribution of individuals to the maintenance of *M. bovis* infection in the population, the following key points are important. Infectious females continue to breed successfully in the face of excretion. The cubs born and reared in social groups with these females are at increased risk of acquiring infection and of that infection progressing during their first year. However, these cubs are only a small proportion of those born each year, and have higher mortality rates. In addition, although movement between social groups has been recorded in cubs, most tend to remain in their natal groups until adulthood, consistent with movement between social groups being motivated largely by breeding behaviour (Rogers *et al.* 1998).

Hence onward transmission of infection by cubs is likely to be to other members of the social group, rather than dissemination outside the social group, resulting in the observed clustering of infection at a social group level (Cheeseman *et al.* 1988, Delahay *et al.* 2000a).

7.6 Implications for management strategies

Breaking the cycle of pseudo-vertical transmission would be a clear goal for any strategy to manage *M. bovis* in badger populations. In this context, it could be that a combination of targeting seropositive breeding females for removal, and targeting other individuals, especially cubs, for vaccination may be viable options. From a practical perspective, the Stat-Pak is the only diagnostic test appropriate for field use. In addition, its sensitivity increases with infection progression (Chambers *et al.* 2009), hence any SP positive individuals could be considered potentially infectious. Targeting of adult females in the context of a strategy of testing and removing all SP positive badgers would contribute to breaking the cycle of pseudo-vertical transmission.

The optimal time for such an approach would be when trapping success is still high during the summer, but there is a minimal chance of the capture of females with dependent cubs. In a previous study of badger culling data, it was suggested that a closed season running from February 1st to April 30th was effective in minimising the welfare risk of the removal of adult females with unweaned cubs (Woodroffe *et al.* 2005). However, any culling strategy carries a risk of social perturbation, which could limit its potential success. Modelling outcomes in populations with varying density and *M. bovis* prevalence would be an essential pre-requisite to the evaluation of such a strategy.

The success of a badger vaccination strategy against *M. bovis* depends on the efficacy of the vaccine in the individual, and delivery of vaccine to a sufficient proportion of susceptible badgers (by definition, prior to the acquisition of infection), to induce herd immunity, protecting the unvaccinated proportion of the population through reducing the quantity and frequency of mycobacterial excretion in the vaccinated population (Blancou *et al.* 2009). A key aim would therefore be to target susceptible cubs as early in their life as possible so as to maximise the chances of vaccine delivery prior to the acquisition of infection. Interestingly, the success of Classical Swine Fever vaccination of wild boar (*Sus scrofa*) in Europe, has been limited by poor bait uptake by juveniles (Rossi *et al.* 2010). This illustrates the complexity of implementation of an effective disease management strategy in wildlife, despite successful development of an effective oral vaccine.

The analyses reported in the present study demonstrated that the majority of cubs did not acquire infection during their first year. However, of those that did, a substantial

proportion were likely to be infected at or close to emergence. Using current methods, vaccine can only be delivered parenterally to cubs captured above ground. The spatial clustering of cubs that acquire infection prior to emergence, however, would minimise the number of social groups to which this limitation applies. The data reported here suggest that it may be a practical option to target cubs for vaccination, but more specific evaluation of the potential success of this strategy or indeed one combined with selective culling of seropositive adult females would require statistical modelling, perhaps using data from these studies to populate the models.

The findings of this thesis contribute to our overall understanding of the effects of *M. bovis* infection in individual badgers, and how these differ between the sexes. In addition, the role of the infectious breeding female badger in the maintenance of infection in this population has been highlighted. Application of these findings will enhance understanding of transmission dynamics at the group and population level and inform the development of future disease control strategies. Analysis of the temporal progression of cell-mediated responses has revealed new information regarding the immunopathogenesis of *M. bovis* infection in badgers, which may have applications for future research programmes.

REFERENCES

Abate, O., Zanatta, R., Malsiano, T. and Dotta, U. (2000) Canine serum protein patterns using high-resolution electrophoresis (HRE). *The Veterinary Journal* **159**, 154-160.

Agranoff, D., Fernandez-Reyes, D., Papadopoulos, M.C., Rojas, S.A., Herbster, M., Loosemore, A., Tarelli, E., Sheldon, J., Schwenk, A., Pollok, R., Rayner, C.F.J. and Krishna, S. (2006) Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. *The Lancet* **368**, 1012-1021.

Amadori, A., Zamarchi, R., De Silvestro, G., Forza, G., Cavatton, G., Danielli, G.A., Clementi, M. and Chieco-Bianchi, L. (1995) Genetic control of the CD4/CD8 T-cell ratio in humans. *Nature Medicine* **1**, 1279-1283.

Anderson, R.M. and May, R.T. (1978) Regulation and stability of host-parasite population interactions. *Journal of Animal Ecology* **47**, 219-247.

Anderson, R.M. and Trewhella, W. (1985) Population dynamics of the badger (*Meles meles*) and the epidemiology of bovine tuberculosis (*Mycobacterium bovis*). *Philosophical Transactions of the Royal Society of London Series B* **310**, 327-381.

Anwar, M.A., Newman, C., Macdonald, D.W., Woolhouse, M.E.J. and Kelly, D.W. (2000) Coccidiosis in the European badger (*Meles meles*) from England, an epidemiological study. *Parasitology* **120**, 255-260.

Anwar, A., Knaggs, J., Service, K.M., McLaren, G.W., Riordan, P., Newman, C., Delahay, R.J., Cheeseman, C. and Macdonald, D.W. (2006) Antibodies to *Toxoplamsa gondii* in Eurasian badgers. *Journal of Wildlife Diseases* **42**, 13-15.

Bentwich, Z., Kalinkovich, A., Weisman, Z., Borkow, G., Beyers, N. and Beyers, A.D. (1999) Can eradication of helminthic infections change the face of AIDS and tuberculosis? *Immunology Today* **11**, 485-487.

Blancou, J., Artois, M., Gilot-Fromont, E., Kaden, V., Rossi, S., Smith, G.C., Hutchings, M.R., Chambers, M.A., Houghton, S. and Delahay, R.J. (2009) In: Delahay, R.J., Smith, G.C. and Hutchings, M.R. (Eds), *Management of Disease in Wild Mammals.* Springer, Tokyo. pp. 97-120.

Böhm, M., Hutchings, M.R. and White, P.C.L. (2009) Contact networks in a wildlife-livestock host community: identifying high-risk individuals in the transmission of bovine TB among badgers and cattle. *Plos One* **4**, e5016.

Boumann, A., Heinemann, M.J. and Faas, M.M. (2005) Sex hormones and the immune response in humans. *Human Reproduction Update* **11**, 411-423.

Bourne, F.J., Donnelly, C., Cox D., Gettinby, G., McInerney, J., Morrison, I. & Woodroffe, R. (2007) Bovine TB: The Scientific Evidence, A Science Base for a Sustainable Policy to Control TB in Cattle, An Epidemiological Investigation into Bovine Tuberculosis. *Final Report of the Independent Scientific Group on Cattle TB*. London: Department for Environment, Food and Rural Affairs.

Bradley, J.E. and Jackson, J.A. (2008) Measuring immune system variation to help understand host-pathogen community dynamics. *Parasitology* **135**, 807-823.

Breen, R.A.M., Leonard, O., Perrin, F.M.R., Smith, C.J., Bhagani, S., Cropley, I. and Lipman, M.C.I. (2008) How good are systemic symptoms and blood inflammatory markers at detecting individuals with tuberculosis? *International Journal of Tuberculosis and Lung Disease* **12**, 44-49.

Buddle, B.M., Aldwell, F.E., Pfeffer, A., de Lisle, G.W. and Corner, L.A. (1994a) Experimental *Mycobacterium bovis* infection of cattle: Effect of dose of *M. bovis* and pregnancy on immune responses and distribution of lesions. *New Zealand Veterinary Journal* **42**, 167-172.

Buddle, B.M., Aldwell, F.E., Pfeffer, A. and de Lisle, G.W. (1994b) Experimental *Mycobacterium bovis* infection in the brushtail possum (*Trichosurus vulpecula*): pathology, haematology and lymphocyte stimulation responses. *Veterinary Microbiology* **38**, 241-254.

Buddle, B.M., Wedlock, D.N., Denis, M. and Skinner, M.A. (2005) Identification of immune response correlates for protection against bovine tuberculosis. *Veterinary Immunology and Immunopathology* **108**, 45-51.

Burke, S.D.A. and Sawchuk, L.A. (2003) Tuberculosis mortality and recent childbirth: a retrospective case-control study of Gibraltarian women, 1874-1884. *Social Science and Medicine* **56**, 477-490.

Cameron, S.J. (1974) Tuberculosis and the blood – a special relationship? *Tubercle* **55**, 55-72.

Carter, S.P., Delahay, R.J., Smith, G.C., Macdonald, D.W., Riordan, P., Etherington, T.R., Pimley, E.R., Walker, N.J. and Cheeseman, C.L. (2007) Culling-induced social perturbation in Eurasian badgers *Meles meles* and the management of TB in cattle: an analysis of a critical problem in applied ecology. *Proceedings of the Royal Society B* **274**, 2769-2777.

Cassidy, J.P. (2006) The pathogenesis and pathology of bovine tuberculosis with insights from studies of tuberculosis in humans and laboratory animal models. *Veterinary Microbiology* **112**, 151-161.

CSL (now FERA) 2007. The long-term intensive ecological and epidemiological investigation of a badger population naturally infected with *M. bovis*. *SID 5 Research Project Final Report to Defra.*

CSL (now FERA) 2008. The long-term intensive ecological and epidemiological investigation of a badger population naturally infected with *M. bovis*. *Interim Report to Defra.*

Cerón, J.J., Eckersall, P.D. and Martińez-Subiela, S. (2005) Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Veterinary Clinical Pathology* **34**, 85-99.

Chambers, M.A., Gavier-Widen, D., Stanley, P.A. and Hewinson, R.G. (2000) Biochemical and haematological parameters associated with tuberculosis in European badgers. *Veterinary Record* **146**, 734-735.

Chambers, M.A., Pressling, W.A., Cheeseman, C.L., Clifton-Hadley, R.S. and Hewinson, R.G. (2002) Value of existing serological tests for identifying badgers that shed *Mycobacterium bovis*. *Veterinary Microbiology* **86**, 183-189.

Chambers, M.A., Rogers, F., Delahay, R.J., Lesellier, S., Ashford, R., Dalley, D., Gowtage, S., Davé, D., Palmer, S., Brewer, J., Crawshaw, T., Clifton-Hadley, R., Carter, S., Cheeseman, C., Hanks, C., Murray, A., Palphramand, K., Pietraville, S., Smith, G.C., Tomlinson, A., Walker, N.J., Wilson, G.J., Corner, L.A.L., Rushton, S.P., Shirley, M.D.F., Gettinby, G., McDonald, R.A. and Hewinson, R.G. (2011) Bacille Calmette-Guérin vaccination reduces the severity of tuberculosis in badgers. *Proceedings of the Royal Society Series B* **278**, 1913-1920.

Chambers, M.A., Crawshaw, T., Waterhouse, S., Delahay, R., Hewinson, R.G. and Layschenko, K.P. (2008) Validation of the BrockTB Stat-Pak assay for detection of tuberculosis in Eurasian badgers (*Meles meles*) and influence of disease severity on diagnostic accuracy. *Journal of Clinical Microbiology* **46**, 1498-1500.

Chambers, M.A., Waterhouse, S., Lyashchenko, K., Delahay, R., Sayers, R. and Hewinson, R.G. (2009) Performance of TB immunodiagnostic tests in Eurasian badgers (*Meles meles*) of different ages and the influence of duration of infection on serological sensitivity. *BMC Veterinary Research* **5**, 42-49.

Chan, T.Y.K., Chan, C.H.S. and Shek, C.C. (1994) The prevalence of hypercalcaemia in pulmonary and miliary tuberculosis – a longitudinal study. *Singapore Medical Journal* **35**, 613-615.

Cheeseman, C.L., Wilesmith, J.W., Ryan, J. and Mallinson, P.J. (1987) Badger population dynamics in a high-density area. *Symposium of the Zoological Society of London* **58**, 279-294.

Cheeseman, C.L., Wilesmith, J.W., Stuart, F.A. and Mallinson, P.J. (1988) Dynamics of tuberculosis in a naturally infected badger population. *Mammal Review* **18**(1), 61-72.

Cheeseman, C.L., Wilesmith, J.W. and Stuart, F.A. (1989) Tuberculosis: the disease and its epidemiology in the badger, a review. *Epidemiology and Infection* **103**, 113-125.

Choi, C.M., Kang, C-I., Jeung, W-K., Kim, D-H., Lee, C-H. and Yim, J-J. (2007) Role of the C-reactive protein for the diagnosis of TB among military personnel in South Korea. *International Journal of Tuberculosis and Lung Disease* **11**, 233-236.

Clifton-Hadley, R.S., Wilesmith, J.W. and Stuart, F.A. (1993) *Mycobacterium bovis* in the European badger *(Meles meles)*: epidemiological findings in tuberculous badgers from a naturally infected population. *Epidemiology and Infection* **111**, 9-19.

Clifton-Hadley, R.S., Sayers, A.R. and Stock, M.P. (1995) Evaluation of an ELISA for *Mycobacterium bovis* infection in badgers (*Meles meles*). *Veterinary Record* **137**, 555-558.

Clutton-Brock, T.H., Major, M. and Guinness, F.E. (1985) Population regulation in male and female red deer. *Journal of Animal Ecology* **54**, 831-846.

Clutton-Brock, T.H., Gaynor, D., McIrath, G.M., Maccoll, A.D.C., Kansky, R., Chadwick, P., Manser, M., Skinner, J.D. and Brotherton, P.N.M. (1999) Predation, group size and mortality in a cooperative mongoose, *Suricata suricatta*. *Journal of Animal Ecology* **68**, 672-683.

Coleman, J.D. and Cooke, M.M. (2001) *Mycobacterium bovis* infection in wildlife in New Zealand. *Tuberculosis* **81**, 191-202.

Collins, H.L. and Kaufmann, S.H.E. (2001) The many faces of host responses to tuberculosis. *Immunology* **103**, 1-9.

Cooper, A.M., Dalton, D.K., Stewart, T.A., Griffin, J.P., Russell, D.G. and Orme, I.M. (1993) Disseminated tuberculosis in interferon γ gene-disrupted mice. *Journal of Experimental Medicine* **178**, 2243-2247.

Corner, L.A.L., Murphy, D. and Gormley, E. (2011) *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*): the disease, pathogenesis, epidemiology and control. *Journal of Comparative Pathology* **144**, 1-24.

Cox, F.E.G. (2001) Concomitant infections, parasites and immune responses. *Parasitology* **122**, S23-38.

Crawshaw, T.R., Griffiths, I.B. and Clifton-Hadley, R.S. (2008) Comparison of a standard and a detailed post-mortem protocol for detecting *Mycobacterium bovis* in badgers. *Veterinary Record* **163**, 473-477.

Cresswell, W.J. and Harris, S. (1988) Foraging behaviour and home-range utilisation in a suburban badger (*Meles meles*) population. *Mammal Review* **18**(1), 37-49.

Cresswell, W.J., Harris, S., Cheeseman, C.L. and Mallinson, P.J. (1992) To breed or not to breed: an analysis of the social and density-dependent constraints on the fecundity of female badgers (*Meles meles*). *Philosophical Transactions of the Royal Society of London Series B* **338**, 393-407.

Cross, P.C., Lloyd-Smith, J.O., Bowers, J.A., Hay, C.T., Hofmeyr, M. and Getz, W.M. (2004) Integrating association data and disease dynamics in a social ungulate: bovine tuberculosis in African buffalo in the Kruger National Park. *Annales Zoologici Fennici* **41**, 879-892.

Cross, P.C., Drewe, J., Patrek, V., Pearce, G., Samuel, M.D. and Delahay, R.J. (2009) Wildlife population structure and parasite transmission: implications for disease management. In: Delahay, R.J., Smith, G.C. and Hutchings, M.R. (Eds), *Management of Disease in Wild Mammals.* Springer, Tokyo. pp. 9-29.

Cruz, A.T. and Starke, J.R. (2007) Clinical manifestations of tuberculosis in children. *Paediatric Respiratory Reviews* **8**, 107-117.

Dai, G. and McMurray, D.N. (1998) Altered cytokine production and impaired antimycobacterial immunity in protein-malnourished guinea pigs. *Infection and Immunity* **66**, 3562-3568.

Dale, T.D. (2005) Gastro-intestinal parasites found in a population of Eurasian badgers (*Meles meles* L. 1758) in Woodchester Park, south west England: A Coprological Study. *MSc thesis unpublished.*

Dalley, D., Chambers, M.A., Cockle, P., Pressling, W., Gavier-Widen, D. and Hewinson, R.G. (1999) A lymphocyte transformation assay for the detection of *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*). *Veterinary Immunology and Immunopathology* **70**, 85-94.

Dalley, D.J., Hogarth, P.J., Hughes, S., Hewinson, R.G. and Chambers, M.A. (2004) Cloning and sequencing of badger (*Meles meles*) interferon γ and its detection in badger lymphocytes. *Veterinary Immunology and Immunopathology* **101**, 19-30.

Dalley, D.J., Dave, D., Lesellier, S., Palmer, S., Crawshaw, T., Hewinson, R.G. and Chambers, M.A. (2008) Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). *Tuberculosis* **88**, 235-243.

Daruna, J. (2004) *Immune System Basics in: Introduction to Psychoneuroimmunology.* Elsevier Academic Press, London.

Day, M.J. (2007) Immune system development in the dog and cat. *Journal of Comparative Pathology* **137**, S10-S15.

Dean, G.S., Rhodes, S.G., Coad, M., Whelan, A.O., Cockle, P.J., Clifford, D.J., Hewinson, R.G. and Vordermeier, H.M. (2005) Minimum infective dose of *Mycobacterium bovis* in cattle. *Infection and Immunity* **73**, 6467-6471.

De Carvalho, L.P.S., Fischer, S.M., Marrero, J., Nathan, C., Ehrt, S. and Rhee, K.Y. (2010) Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalised co-catabolism of carbon substrates. *Chemistry and Biology* **17**, 1122-1131.

Defra (2011a). Breakdown of bovine TB expenditure from the England bTB Programme budget. Accessed on 03 August 2011 via: http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/tb/documents/expenditure-stats.pdf

Defra (2011b). Next steps to tackle bovine TB in England. Accessed on 03 August 2011 via: http://www.defra.gov.uk/news/2011/07/19/next-steps-to-tackle-bovine-tb-in-england-2/

Defra (2011c). Badger vaccine deployment project. Accessed on 03 August 2011 via: http://www.fera.defra.gov.uk/wildlife/ecologyManagement/bvdp/

Delahay, R.J., Langton, S., Smith, G.C., Clifton-Hadley, R.S. and Cheeseman, C.L. (2000a) The spatio-temporal distribution of *Mycobacterium bovis* (bovine tuberculosis) infection in a high-density badger population. *Journal of Animal Ecology* **69**, 428-441.

Delahay, R.J., Brown, J.A., Mallinson, P.J., Spyvee, P.D., Handoll, D., Rogers, L.M. and Cheeseman, C.L. (2000b) The use of marked bait in studies of the territorial organisation of the European badger (*Meles meles*). *Mammal Review* **30**, 73-87.

Delahay, R.J., Walker, N., Forrester, G.J., Harmsen, B., Riordan, P., Macdonald, D.W., Newman, C. and Cheeseman, C.L. (2006a) Demographic correlates of bite wounding in Eurasian badgers, *Meles meles* L., in stable and perturbed populations. *Animal Behaviour* **71**, 1047-1055.

Delahay, R.J., Carter, S.P., Forrester, G.J., Mitchell, A. and Cheeseman, C.L. (2006b) Habitat correlates of group size, bodyweight and reproductive performance in a high-density Eurasian badger (*Meles meles*) population. *Journal of Zoology* **270**, 437-447.

De Leeuw, A.N.S., Forrester, G.J., Spyvee, P.D., Brash, M.G.I, and Delahay, R.J. (2004) Experimental comparison of ketamine with a combination of ketamine, butorphanol and medetomidine for general anaesthesia of the Eurasian badger (*Meles meles* L.) *The Veterinary Journal* **167**, 186-193.

De Lisle, G.W., Mackintosh, C.G. and Bengis, R.G. (2001) *Mycobacterium bovis* in free-living and captive wildlife, including farmed deer. *Scientific and Technical Review. OIE* **20**, 86-111.

Dockerty, R. (2007) The prevalence of non-typhoidal *Salmonellae* in UK badgers: significance and epidemiology. *MSc thesis unpublished.*

Doherty, T.M., Demissie, A., Olobo, J., Wolday, D., Britton, S., Eguale, T., Ravn, P. and Andersen, P. (2002) Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal and subclinical infection among contacts of tuberculosis patients. *Journal of Clinical Microbiology* **40**, 704-706.

Domingo-Roura, X., Newman, C., Calafell, F. and Macdonald, D.W. (2001) Blood biochemistry reflects seasonal nutritional and reproductive constraints in the Eurasian badger (*Meles meles*). *Physiological and Biochemical Zoology* **74**, 450-460.

Donnelly, C.A., Woodroffe, R., Cox, D.R., Bourne, J., Gettinby, G., le Fevre, A.M., McInerney, J.P. and Morrison, W.I. (2003) Impact of localised badger culling on tuberculosis incidence in British cattle. *Nature* **426**, 834-837.

Donnelly, C.A., Wei, G., Johnston, W.T., Cox, D.R., Woodroffe, R., Bourne, F.J., Cheeseman, C.L., Clifton-Hadley, R.S., Gettinby, G., Gilks, P., Jenkins, H.E., LeFevre, A.M., McInerney, J.P. and Morrison, W.I. (2007) Impacts of widespread badger culling on cattle tuberculosis: concluding analyses from a large-scale field trial. *International Journal of Infectious Diseases* **11**, 300-308.

Drewe, J.A. (2010) Who infects whom? Social networks and tuberculosis transmission in meerkats. *Proceedings of the Royal Society, Series B* **277**, 633-642.

Drewe, J.A., Tomlinson, A.J., Walker, N.J. and Delahay, R.J. (2010) Diagnostic accuracy and optimal use of three tests for tuberculosis in live badgers. *PLoS One* **5**, e11196

Eckersall, P.D. (2006) Acute phase proteins as biomarkers in companion and laboratory animals. *Proceedings of the Annual Meeting of the American College of Veterinary*

Pathologists and American Society for Veterinary Clinical Pathology, Tuscon, Arizona. Accessed via ivis.org.

Eideinger, D. and Garrett, T.J. (1972) Studies of the regulatory effects of the sex hormones on antibody formation and stem cell differentiation. *The Journal of Experimental Medicine* **136**, 1098-1116.

Elias, D., Wolday, D., Akuffo, H., Petros, B., Bronner, U. and Britton, S. (2001) Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guérin (BCG) vaccination. *Clinical and Experimental Immunology* **123**, 219-225.

Elias, D., Akuffo, H. and Britton, S. (2005) PPD induced in vitro interferon gamma production is not a reliable correlate of protection against *Mycobacterium tuberculosis*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**, 363-368.

Elias, D., Britton, S., Aseffa, A., Engers, H. and Akuffo, H. (2008) Poor immunogenicity of BCG in helminth infected population is associated with increased *in vitro* TGF-β production. *Vaccine* **26**, 3897-3902.

Fenton, A., Lamb, T. and Graham, A.L. (2008) Optimality analysis of Th1/Th2 immune responses during micro-parasite-macroparasite co-infection, with epidemiological feedbacks. *Parasitology* **135**, 841-853.

FERA (2011) The long-term intensive ecological and epidemiological investigation of a badger population naturally infected with *M. bovis*. *SID 5 Research Project Final Report to Defra*.

Fletcher, H.A. (2007) Correlates of immune protection from tuberculosis. *Current Molecular Medicine* **7**, 319-325.

Flynn, J.L. and Chan, J. (2001) Immunology of tuberculosis. *Annual Reviews in Immunology* **19**, 93-129.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A. and Bloom, B.R. (1993) An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *Journal of Experimental Medicine* **178**, 2249-2254.

Flynn, R.J., Mulcahy, G., Welsh, M., Cassidy, J.P., Corbett, D., Milligan, C., Andersen, P., Strain, S. and McNair, J. (2009) Co-infection of cattle with *Fasciola hepatica* and *Mycobacterium bovis* – immunological consequences. *Transboundary and Emerging Diseases* **56**, 269-274.

Forrester, G.J., Delahay, R.J. and Clifton-Hadley, R.S. (2001) Screening badgers (*Meles meles*) for *Mycobacterium bovis* infection by using multiple applications of an ELISA. *Veterinary Record* **149**, 169-172.

Fountain, J.R. (1954) Blood changes associated with disseminated tuberculosis. Report of four fatal cases and review. *British Medical Journal* **2**, 76-79.

Gallagher, J., Macadam, I., Sayer, J. and van Lavieren, L.P. (1972) Pulmonary tuberculosis in free-living lechwe antelope in Zambia. *Tropical Animal Health Production* **4**, 204-213.

Gallagher, J., Muirhead, R.H. and Burn, K.J. (1976) Tuberculosis in wild badgers (*Meles meles*) in Gloucestershire: Pathology. *Veterinary Record* **98**, 9-14.

Gallagher, J. and Nelson, J. (1979) Cause of ill health and natural death in badgers in Gloucestershire. *Veterinary Record* **105**, 546-551.

Gallagher, J., Monies, R., Gavier-Widen, M. and Rule, B. (1998) Role of infected, non-diseased badgers in the pathogenesis of tuberculosis in the badger. *Veterinary Record* **142**, 710-714.

Gallagher, J. and Clifton-Hadley, R.S. (2000) Tuberculosis in badgers; a review of the disease and its significance for other animals. *Research in Veterinary Science* **69**, 203-217.

Gallagher, J., Muirhead, R.H., Turnbull, A.T., Davies, J.I., Ashton, W.L.G., Smith, J., Daykin, J. and McDiarmid, A. (2006) TB policy and the badger culling trials. *Veterinary Record* **158**, 310-311.

Gavier-Widen, D., Chambers, M.A., Palmer, N., Newell, D.G. and Hewinson, R.G. (2001) Pathology of natural *Mycobacterium bovis* infection in European badgers (*Meles meles*) and its relationship with bacterial excretion. *Veterinary Record* **148**, 299-304.

Girón-González, J.A., Moral, F.J., Elvira, J., García-Gil, D., Guerrero, F., Gavilán, I. and Escobar, L. (2000) Consistent production of a higher Th_1:Th_2 cytokine ratio by stimulated T cells in men compared with women. *European Journal of Endocrinology* **143**, 31-36.

Glatman-Freedman, A. (2006) The role of antibody-mediated immunity in defence against *Mycobacterium tuberculosis*: advances towards a novel vaccine strategy. *Tuberculosis* **86**, 191-197.

Gloucestershire Wildlife Trust (2011). Badger bovine TB vaccination due to start in June. Accessed on 03 August via: http://www.gloucestershirewildlifetrust.co.uk/Gwt/Gwt.Nsf/WEBARTICLE?OpenForm&id=E454586FE08C76D880257878005698F)

Goodger, J., Nolan, A., Russell, W.P., Dalley, D.J., Thorns, C.J., Stuart, F.A., Croston, P. and Newell, D.G. (1994) Serodiagnosis of *Mycobacterium bovis* infection in badgers: development of an indirect ELISA using a 25kDa antigen. *Veterinary Record* **135**, 82-85.

Greenwald, R., Esfandiari, J., Lesellier, S., Houghton, R., Pollock, J., Aagaard, C., Andersen, P., Hewinson, R.G., Chambers, M.A. and Lyashchenko, K. (2003) Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. *Diagnostic Microbiology and Infectious Diseases* **46**, 197-203.

Hamadeh, M.A. and Glassroth, J. (1992) Tuberculosis and pregnancy. *Chest* **101**, 1114-1120.

Hancox, M. (1980) Parasites and infectious diseases of the Eurasian badger (*Meles meles*): a review. *Mammal Review* **10(4)**, 151-162.

Hardie, R.M. and Watson, J.M. (1992) *Mycobacterium bovis* in England and Wales: past, present and future. *Epidemiology and Infection* **109**, 23-33.

Hayne, D.W. (1949) Calculation of size of home range. *Journal of Mammalogy* **30**, 1-18.

Harris, S. and Cresswell, W.J. (1987) Dynamics of a suburban badger (*Meles meles*) population. *Symposia of the Zoological Society of London* **58**, 295-311.

Hoft, D. (2008) Tuberculosis vaccine development: goals, immunological design and evaluation. *Lancet* **372**, 164-175.

Horne, D.J., Hubbard, R., Narita, M., Exarchos, A., Park, D.R. and Goss, C.H. (2010) Factors associated with mortality in patients with tuberculosis. *BMC Infectious Diseases* **10**, 258-265.

Houlihan, M.G., Dixon, F.W. and Page, N.A (2008) Outbreak of bovine tuberculosis featuring anergy to the skin test, udder lesions and milkborne disease in young calves. *Veterinary Record* **163**, 357-361.

Janeway, C.A., Travers, P., Walport, M. and Shlomchik, M.J. (2005) *Immunobiology – the immune system in health and disease.* Garland Science Publishing: New York.

Jenkins, H.E., Woodroffe, R. and Donnelly, C.A. (2008a) The effects of annual widespread badger culls on cattle tuberculosis following the cessation of culling. *International Journal of Infectious Diseases* **12**, 457-465.

Jenkins, H.E., Morrison, W.I., Cox, D.R., Donnelly, C.A., Johnston, W.T., Bourne, F.J., Clifton-Hadley, R.S., Gettinby, G., McInerney, J.P., Watkins, G.H. and Woodroffe, R. (2008b) The prevalence, distribution and severity of detectable pathological lesions in badgers naturally infected with *Mycobacterium bovis*. *Epidemiology and Infection* **136**, 1350-1361.

Jenkins, H.E., Woodroffe, R. and Donnelly, C.A. (2010) The duration of the effects of repeated widespread badger culling on cattle tuberculosis following the cessation of culling. *Plos One* **5**, e9090.

Jolles, A.E., Cooper, D. and Levin, S.A. (2005) Hidden effects of chronic tuberculosis in African buffalo. *Ecology* **86**, 2258-2364.

Jolles, A.E., Ezenwa, V.O., Etienne, R.S., Turner, W.C. and Olff, H. (2008) Interactions between macroparasites and microparasites drive infection patterns in free-ranging African Buffalo. *Ecology* **89**, 2239-2250.

Jones, G.W., Neal, C. and Harris, E.A. (1980) The helminth parasites of the badger (*Meles meles*) in Cornwall. *Mammal Review* **10(4)**, 163-164.

Karlson, A.G. and Lessel, E.F. (1970) *Mycobacterium bovis* Nom. Nov. *International Journal of Systematic Bacteriology* **20** (3), 273-282.

Kaufmann, S.H.E. and Parida, S.K. (2008) Tuberculosis in Africa: Learning from pathogenesis for biomarker identification. *Cell Host and Microbe* **4**, 219-228.

Keeling, M.J. (1999) The effects of local spatial structure on epidemiological invasions. *Proceedings of the Royal Society of London, Series B* **266**, 859-867

Kerr, M.G. (1989) Veterinary Laboratory Medicine. *Clinical biochemistry and haematology.* Blackwell Scientific Publications, Oxford.

King, D. (2007). Bovine tuberculosis in cattle and badgers. *A report by the Chief Scientific Adviser, Sir David King.* Accessed via on 18 September 2008 via: http://www.defra.gov.uk/animalh/tb/pdf/badgersreport-king.pdf

King, D.P., Mutukwa, M., Lesellier, S., Cheeseman, C.L., Chambers, M.A. and Banks, M. (2004) Detection of mustelid herpesvirus-1 infected European badgers (*Meles meles*) in the British Isles. *Journal of Wildlife Diseases* **40**, 99-102.

Kirkwood, J.K., Sainsbury, A.W. and Bennett, P.M. (1994) The welfare of free-living wild animals: methods of assessment. *Animal Welfare* **3**, 257-273.

Klein, S.L. (2004) Hormonal and immunological mechanisms mediating sex differences in parasite infection. *Parasite Immunology* **26**, 247-264.

Kotlan, A. and Pospesch, L. (1933) Coccidial infections in the badger *Meles taxus* L. *Parasitology* **25**, 102-107.

Krebs, J.R. and the Independent Scientific Review Group. (1997) Bovine Tuberculosis in Cattle and Badgers. *Report to the Right Honourable Dr. Jack Cunningham, MP.* HMSO, London.

Kruuk, H. (1978) Spatial organisation and territorial behaviour of the European badger *Meles meles*. *Journal of Zoology* **184**, 1-19.

Kruuk, H. and Parish, T. (1981) Feeding specialisation of the European badger *Meles meles* in Scotland. *Journal of Animal Ecology* **50**, 773-788.

Kruuk, H. and Parish, T. (1983) Seasonal and local differences in the weight of European badgers (*Meles meles* L.) in relation to food supply. *Sonderdruck aus Z. f Saugetierkunde Bd.* **48**, 45-50.

Kruuk, H., Conroy, J.W.H. and Moorhouse, A. (1987) Seasonal reproduction, mortality and food of otters (*Lutra lutra* L.) in Shetland. *Symposia of the Zoological Society of London* **58**, 263-278.

Kwan, C.K. and Ernst, J.D. (2011) HIV and tuberculosis: a deadly human syndemic. *Clinical Microbiology Reviews* **24**, 351-376.

Lalvani, A. and Pareek, M. (2009) A 100 year update on diagnosis of tuberculosis infection. *British Medical Bulletin* **93**, 69-84.

Le Cren, E.D. (1951) The length-weight relationship and seasonal cycle in gonad weight and condition in the perch (*Perca fluviatilis*). *Journal of Animal Ecology* **20**, 201-219.

Lello, J. and Hussell, T. (2008) Functional group/guild modelling of inter-specific pathogen interactions: A potential tool for predicting the consequences of co-infection. *Parasitology* **135**, 825-839.

Lepper, A.W.D., Pearson, C.W. and Corner, L.A.L. (1977) Anergy to tuberculin in beef cattle. *Australian Veterinary Journal* **53**, 214-216.

Lesellier, S. (2007) 'Immunological responses of European badgers (*Meles meles*) vaccinated with BCG and/or experimentally challenged with *Mycobacterium bovis*'. PhD thesis, University College of Dublin.

Lesellier, S., Corner, L., Costello, E., Sleeman, P., Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Hewinson, R.G., Chambers, M. and Gormley, E. (2009) Immunological responses following experimental endobronchial infection of badgers (*Meles meles*) with different doses of *Mycobacterium bovis*. *Veterinary Immunology and Immunopathology* **127**, 174-180.

Lesellier, S., Palmer, S., Gowtage-Sequiera, S., Ashford, R., Dalley, D., Davé, D., Weyer, U., Salguero, F.J., Nunez, A., Crawshaw, T., Corner, L.A.L., Hewinson, R.G. and Chambers, M.A. (2011) Protection of Eurasian badgers (*Meles meles*) from tuberculosis after intra-muscular vaccination with different doses of BCG. *Vaccine* **29**, 3782-3790.

Liebeschuetz, S., Bamber, S., Ewer, K., Deeks, J., Pathan, A.A. and Lalvani, A. (2004) Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. *Lancet* **364**, 2196-2203.

Little, T.W.A., Naylor, P.F. and Wilesmith, J.W. (1982) Laboratory study of *Mycobacterium bovis* infection in badgers and calves. *Veterinary Record* **111**, 550-557.

Lloyd-Smith, J.O., Schreiber, S.J., Kopp, P.E. and Getz, W.E. (2005) Superspreading and the effect of individual variation on disease emergence. *Nature* **438**, 355-359.

Lurie, M.B. and Dannenberg, A.M. (1965) Macrophage function in infectious disease with inbred rabbits. *Bacteriological Reviews* **29**, 466-476.

Macallan, D.C. (1999) Malnutrition in tuberculosis. *Diagnostic Microbiology and Infectious Disease* **34**, 153-157.

Maher, J., Kelly, P., Hughes, P. and Clancy, L. (1992) Skin anergy and tuberculosis. *Respiratory Medicine* **86**, 481-484.

Mahmood, K.H., Stanford, J.L., Rook, G.A.W., Stuart, F.A., Pritchard, D.G. and Brewer, J.I. (1987a) The immune responses in two populations of wild badgers naturally infected with bovine tubercle bacilli. *Tubercle* **68**, 119-125.

Mahmood, K.H., Rook, G.A.W., Stanford, J.L., Stuart, F.A. and Pritchard, D.G. (1987b) The immunological consequences of challenge with bovine tubercle bacilli in badgers (*Meles meles*). *Epidemiology and Infection* **98**, 155-163.

Mahmood, K.H., Stanford, J.L., Machin, S., Watts, M., Stuart, F.A. and Pritchard, D.G. (1988) The haematological values of European badgers (*Meles meles*) in health and in the course of tuberculosis infection. *Epidemiology and Infection* **101**, 231-237.

Maurel, D. and Boissin, J. (1979) Seasonal variations of thyroid activity in the adult male badger, (*Meles meles* L.). *General and Comparative Endocrinology* **38**, 207-214.

Michel, A.L., Coetzee, M.L., Keet, D.F., Maré, L., Warren, R., Cooper, D., Bengis, R.G., Kremer, K. and van Helden, P. (2009) Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South Africa game reserves. *Veterinary Microbiology* **133**, 335-343.

Miller, L.D., Thoen, C.O., Throlson, K.J., Himes, E.M. and Morgan, R.L. (1989) Serum biochemical and haematologic values of normal and *Mycobacterium bovis* infected American bison. *Journal of Veterinary Diagnostic Investigation* **1**, 219-222.

Mistry, R., Cliff, J.M., Clayton, C.L., Beyers, N., Mohamed, Y.S., Wilson, P.A., Dockrell, H.M., Wallace, D.M., van Helden, P.D., Duncan, K. and Lukey, P.T. (2007) Gene-expression

patterns in whole blood identify subjects at risk for recurrent tuberculosis. *Journal of Infectious Diseases* **195**, 357-365.

Mohr, C.O. (1947) Table of equivalent populations of North American small mammals. *American Midland Naturalist* **37**, 223-249.

Morris, J.A., Stevens, A.E., Little, T.W.A. and Stuart, P. (1978) Lymphocyte unresponsiveness to PPD tuberculin in badgers infected with *Mycobacterium bovis*. *Research in Veterinary Science* **25**, 390-392.

Muirhead, R.H., Gallagher, J. and Burn, K.J. (1974) Tuberculosis in wild badgers in Gloucestershire: Epidemiology. *Veterinary Record* **95**, 552-555.

Munroe, F.A., Dohoo, I.R. and McNab, W.B. (2000) Estimates of within-herd incidence rates of *Mycobacterium bovis* in Canadian cattle and cervids between 1985 and 1994. *Preventive Veterinary Medicine* **45**, 247-256.

Murphy, D., Gormley, E., Costello, E., O'Meara, D. and Corner, L.A.L. (2010) The prevalence and distribution of *Mycobacterium bovis* infection in European badgers (*Meles meles*) as determined by enhanced post mortem examination and bacteriological culture. *Research in Veterinary Science* **88**, 1-5.

Naranjo, V., Gortazar, C., Vicente, J. and de la Fuente, J. (2008) Evidence of the role of the European wild boar as a reservoir of *Mycobacterium tuberculosis* complex. *Veterinary Microbiology* **127**, 1-9.

National Trust (2011). New programme to demonstrate badger vaccination as effective alternative in bovine TB control. Accessed on 03 August 2011 via: http://www.nationaltrust.org.uk/main/w-global/w-news/w-latest_news/w-news-four-year-badger-vaccination-programme.htm

Neal, E. and Cheeseman, C. (1996) *Badgers*. T and AD Poyser Ltd., London.

Newell, D.G., Clifton-Hadley, R.S. and Cheeseman, C.L. (1997) The kinetics of serum antibody responses to natural infections with *Mycobacterium bovis* in one badger social group. *Epidemiology and Infection* **118**, 173-180.

Newman, S.H., Anderson, D.W., Ziccardi, M.H., Trupkiewicz, J.G., Tseng, F.S., Christopher, M.M. and Zinkl, J.G. (2000) An experimental soft-release of oil-spill rehabilitated American coots (*Fulica Americana*): II. Effects on health and blood parameters. *Environmental Pollution* **107**, 295-304.

Newman, C., Macdonald, D.W. and Anwar, M.A. (2001) Coccidiosis in the European badger, *Meles meles* in Wytham Woods: infection and consequences for growth and survival. *Parasitology* **123**, 133-142.

Olea-Popelka, F.J., Griffin, J.M., Collins, J.D., McGrath, G. and Martin, S.W. (2003) Bovine tuberculosis in badgers in four areas in Ireland: does tuberculosis cluster? *Preventive Veterinary Medicine* **59**, 103-111.

Orme, I.M. (2004) Adaptive immunity to mycobacteria. *Current Opinion in Microbiology* **7**, 58-61.

Palmer, M.V., Whipple, D.L., Payeur, J.B., Alt, D.P., Esch, K.J., Bruning-Fann, C.S. and Kaneene, J.B. (2000) Naturally occurring tuberculosis in white-tailed deer. *Journal of American Veterinary Medical Association* **216**, 1921-1924.

Peirce, M.A. and Neal, C. (1973) *Trypanosoma (Megatrypanum) pestanai* in British badgers (*Meles meles*). *International Journal for Parasitology* **4**, 439-440.

Peirce, M.A. and Neal, C. (1974) Piroplasmosis in British badgers (*Meles meles*). *Veterinary Record* **94**, 493-494.

Pellérdy, L. (1955) Beitrage zur Kenntnis der coccidian des Dachses (*Meles taxus*). *Acta Veterinaria* **5**, 421-424.

Petersen, H.H., Nielsen, J.P. and Heegaard, P.M.H. (2004) Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary Research* **35**, 163-187.

Pritchard, D.G., Stuart, F.A., Wilesmith, J.W., Cheeseman, C.L., Brewer, J.I., Bode, R. and Sayers, P.E. (1986) Tuberculosis in East Sussex III. Comparison of post-mortem and clinical methods for the diagnosis of tuberculosis in badgers. *Journal of Hygiene Cambridge* **97**, 27-36.

Pritchard, D.G., Stuart, F.A. and Brewer, J.I. (1987) Experimental infection of badgers (*Meles meles*) with *Mycobacterium bovis*. *Epidemiology and Infection* **98**, 145-154.

Raby, E., Moyo, M., Devendra, A., Banda, J., De Haas, P., Ayles, H. and Godfrey-Faussett, P. (2008) The effects of HIV on the sensitivity of a whole blood IFN-γ release assay in Zambian adults with active tuberculosis. *Plos One* **3**, e2489.

Read, J.M., Eames, K.T.D. and Edmunds, W.J. (2008) Dynamic social networks and the implications for the spread of infectious disease. *Journal of the Royal Society Interface* **5**, 1001-1007.

Renwick, A.R., White, P.C.L. and Bengis, R. G. (2007) Bovine tuberculosis in southern African wildlife: a multi-species host-pathogen system. *Epidemiology and Infection* **135**, 529-540.

Resende Co, T., Hirsch, C.S., Toossi, Z., Dietze, R. and Ribeiro-Rodrigues, R. (2006) Intestinal helminth co-infection has a negative impact on both anti-*Mycobacterium*

tuberculosis immunity and clinical responses to tuberculosis therapy. *Clinical and Experimental Immunology* **147**, 45-52.

Rhodes, S.G., Buddle, B.M., Hewinson, R.G. and Vordermeier, H.M. (2000) Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. *Immunology* **99**, 195-202.

Ritacco, V., Lopez, B., de Kantor, I.N., Barrera, L., Errico, F. and Nader, A. (1991) Reciprocal cellular and humoral immune responses in bovine tuberculosis. *Research in Veterinary Science* **50**, 365-367.

Rodwell, T.C., Whyte, I.J. and Boyce, W.M. (2001) Evaluation of population effects of bovine tuberculosis in free-ranging African buffalo (*Syncerus caffer*). *Journal of Mammalogy* **82**, 231-238.

Rogers, L.M., Cheeseman, C.L. and Mallinson, P.J. (1997) The demography of a high-density badger (*Meles meles*) population in the west of England. *Journal of Zoology* **242**, 705-728.

Rogers, L.M., Delahay, R.J., Cheeseman, C.L., Langton, S., Smith, G.C. and Clifton-Hadley, R.S. (1998) Movement of badgers (*Meles meles*) in a high-density population: individual, population and disease effects. *Proceedings of the Royal Society of London, Series B* **265**, 1269-1276.

Rogers, L.M., Delahay, R.J., Hounsome, T.D. and Cheeseman, C.L. (2000) Changes in badger, *Meles meles*, social organisation in response to increasing population density at Woodchester Park, south-west England. In: Griffiths, H.I. (Ed) *Mustelids in a modern world. Management and conservation aspects of small carnivore: human interactions.* Backhuys Publishers, The Netherlands.

Rook, G.A.W., Dheda, K. and Zumla, A. (2005) Immune responses to tuberculosis in developing countries: implications for new vaccines. *Nature Reviews Immunology* **5**, 661-667.

Rook, G.A.W. (2007) Th2 cytokines in susceptibility to tuberculosis. *Current Molecular Medicine* **7**, 327-337.

Rossi, S., Pol, F., Masse-provin, N., Rigaux, S., Bronner, A. and Le Potier, M-F. (2010) Preventive vaccination contributes to control classical swine fever in wild boar (*Sus scrofa* sp.). *Veterinary Microbiology* **142**, 99-107.

Salt, G.F.H. and Little, T.W.A. (1977) Leptospires isolated from wild mammals caught in the south west of England. *Research in Veterinary Science* **22**, 126-127.

Sawyer, J., Mealing, D., Dalley, D., Dave, D., Lesellier, S., Palmer, S., Bowen-Davies, J., Crawshaw, T.R. and Chambers, M.A. (2007) Development and evaluation of a test for tuberculosis in live European badgers (*Meles meles*) based on measurement of gamma interferon mRNA by real-time PCR. *Journal of Clinical Microbiology* **45**, 2398.

Scanlon, M.P. and Quinn, P.J. (2000) Inactivation of *Mycobacterium bovis* in cattle slurry by five volatile chemicals. *Journal of Applied Microbiology* **89**, 854-861.

Schalk, G. and Forbes, M.R. (1997) Male biases in parasitism of mammals: effects of study type, host age and parasite taxon. *Oikos* **78**, 67-74.

Schuurs, A.H.W.M. and Verheul, H.A.M. (1990) Effects of sex and sex steroids on the immune response. *Journal of Steroid Biochemistry* **35**, 157-172.

Segall, L. and Covic, A. (2010) Diagnosis of tuberculosis in dialysis patients: current strategy. *Clinical Journal of the American Society of Nephrology* **5**, 1114-1122.

Siawaya, J.F.D., Bapela, N.B., Ronacher, K., Veenstra, H., Kidd, M., Gie, R., Beyers, N., van Helden, P. and Walzl, G. (2008) Immune parameters as markers of tuberculosis extent of disease and early prediction of anti-tuberculosis chemotherapy response. *Journal of Infection* **56**, 340-347.

Snider, D. (1984) Pregnancy and tuberculosis. *Chest* **86**, 10S-13S.

Southey, A., Costello, E. and Gormley, E. (2002) Detection of *Mycobacterium bovis* infection and production of interleukin-2 by in vitro stimulation of badger lymphocytes. *Veterinary Immunology and Immunopathology* **87**, 73-78.

Taylor, M.A., Coop, R.L. and Wall, R.L. (2007) *Veterinary Parasitology*. Third Edition. Blackwell Publishing, Oxford.

Telfer, S., Birtles, R., Bennett, M., Lambin, X., Paterson, S and Begon, M. (2008) Parasite interactions in natural populations: insights from longitudinal data. *Parasitology* **135**, 767-781.

Thacker, T.C., Palmer, M.V. and Waters, W.R. (2007) Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle. *Veterinary Immunology and Immunopathology* **119**, 204-213.

Thorns, C.J. and Morris, J.A. (1983) The immune spectrum of *Mycobacterium bovis* infections in some mammalian species: a review. *Veterinary Bulletin* **53**, 543-550.

Turner, J., Rhoades, E.R., Keen, M., Belisle, J.T., Frank, A. and Orme, I.M. (2000) Effective pre-exposure tuberculosis vaccines fail to protect when they are given in an immunotherapeutic mode. *Infection and Immunity* **68**, 1706-1709.

Van Crevel, R., Ottenhoff, T.H.M., and Van der Meer, J.W.M. (2002) Innate immunity to *Mycobacterium tuberculosis*. *Clinical Microbiology Reviews* **15**, 294-309.

Van Riet, E., Hartgens, F.C. and Yazdanbakhsh, M. (2007) Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* **212**, 475-490.

VLA (now AHVLA) (2005). Development of immunological assays for the detection of *Mycobacterium bovis* infection in badgers. *SID 5 Research Project Final Report to Defra*.

VLA/FERA (2010). First tuberculosis vaccine for badgers is authorised. Accessed on 03 August 2011 via: http://vla.defra.gov.uk/news/new_badgerbcg.htm

Vicente, J., Delahay, R.J., Walker, N. and Cheeseman, C.L. (2007a) Social organisation and movement influence the incidence of bovine tuberculosis in an undisturbed high density badger (*Meles meles*) population. *Journal of Animal Ecology* **76**(2), 348-360.

Vicente, J., Hőfle, U., Fernandez-de-Mera, I.G. and Gortazar, C. (2007b) The importance of parasite life history and host density in predicting the impact of infections in red deer. *Oecologia* **152**, 655-664.

Vordermeier, H.M., Chambers, M.A., Cockle, P.J., Whelan, A.O., Simmons, J. and Hewinson, R.G. (2002) Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infection and Immunity* **70**, 3026-3032.

Walzl, G., Ronacher, K., Hanekom, W., Scriba, T.J. and Zumla, A. (2011) Immunological biomarkers of tuberculosis. *Nature Reviews Immunology* **11**, 343-354.

Weinstein, Y., Ran, S. and Segal, S. (1984) Sex-associated differences in the regulation of immune responses controlled by the MHC of the mouse. *The Journal of Immunology* **132**, 656-661.

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G. and Pollock, J.M. (2005) Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology* **114**, 101-111.

Wilkinson, D., Smith, G.C., Delahay, R.J., Rogers, L.M., Cheeseman, C.L. and Clifton-Hadley, R.S. (2000) The effects of bovine tuberculosis (*Mycobacterium bovis*) on mortality in a badger (*Meles meles*) population in England. *Journal of Zoology* **250**, 389-395.

Winnacker, H., Walker, N., Brash, M.G.I., MacDonald, J.A. and Delahay, R.J. (2008) Haematological and biochemical measurements in a population of wild Eurasian badgers (*Meles meles*). *Veterinary Record* **162**, 551-555.

Woodroffe, R. (1993) Alloparental behaviour in the European badger. *Animal Behaviour* **46**, 413-415.

Woodroffe, R. (1995) Body condition affects implantation date in the European badger *Meles meles*. *Journal of Zoology* **236**, 183-188.

Woodroffe, R. and Macdonald, D.W. (1995) Costs of breeding status in the European badger, *Meles meles*. *Journal of Zoology* **235**, 237-245.

Woodroffe, R., Bourne, F.J., Cheeseman, C.L., Cox, D.R., Donnelly, C.A., Gettinby, G., McInerney, J.P. and Morrison, W.I. (2005) Welfare of badgers (*Meles meles*) subjected to culling: development and evaluation of a closed season. *Animal Welfare* **14**, 19-25.

Woodroffe, R., Donnelly, C.A., Cox, D.R., Bourne, F.J., Cheeseman, C.L., Delahay, R.J., Gettinby, G., McInerney, J.P. and Morrison, W.I. (2006) Effects of culling on badger *Meles meles* spatial organization: Implications for the control of bovine tuberculosis. *Journal of Applied Ecology* **43**, 1-10.

Yamamoto, S., Tagata, K., Nagahata, H., Ishikawa, Y., Morimatsu, M. and Naiki, M. (1992) Isolation of C-reactive protein and characterisation of its properties. *Veterinary Immunology and Immunopathology* **30**, 329-339.

Zuk, M. (1990) Reproductive strategies and disease susceptibility: an evolutionary viewpoint. *Parasitology Today* **6**, 231-233.

Appendix: Body condition index derivation

Method

An objective body condition index (BCI) was constructed for badgers captured during the Woodchester Park study from 1997-2009, based on the relationship between body length and weight:

$W=aL^n$

where W = weight, L = body length, a and n = constants (Le Cren 1951).

Linear regression of the natural logarithm (ln) W against ln L allowed estimation of constants separately for cubs, adult males and adult females, in order to calculate a body condition index for each capture event (BCI = observed W / aL^n). These three categories were chosen based on the differing morphological characteristics of each respectively. Hence where BCI >1 the individual weighed more than predicted from their length, and where BCI <1, they weighed less. Figures 1, 2 and 3 show the regression plots for cubs (n=2246) adult males (n=1303) and adult females (n=1779).

(NB – cubs considered to be cubs until March 1st in the year following that of their first capture as cubs).

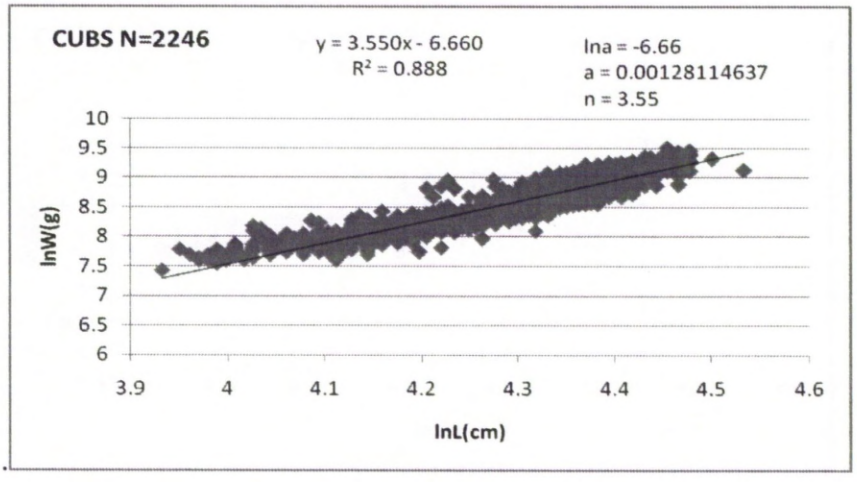

Figure 1. Calculation of a BCI for cubs, captured at Woodchester Park 1997-2009.

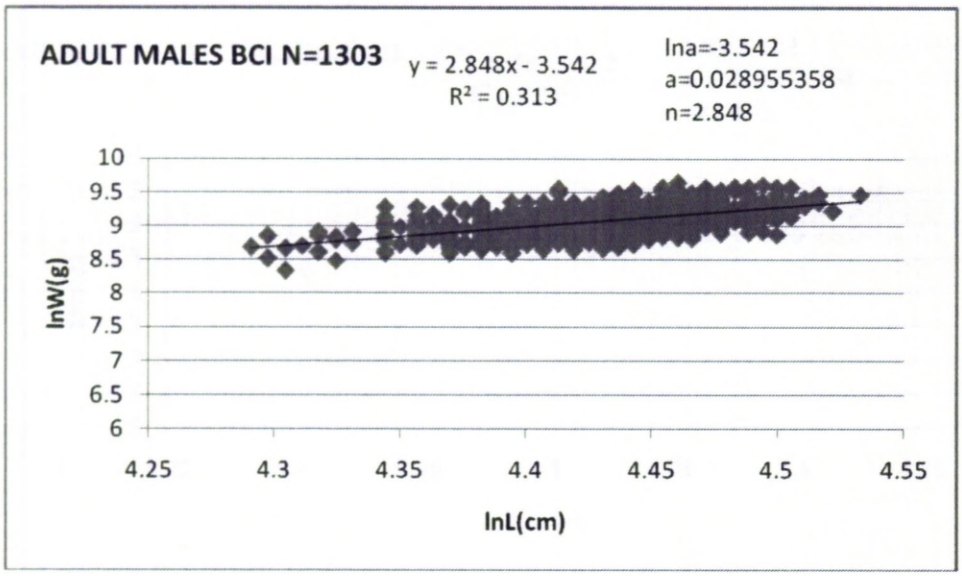

Figure 2. Calculation of a BCI for adult male badgers, captured at Woodchester Park 1997-2009.

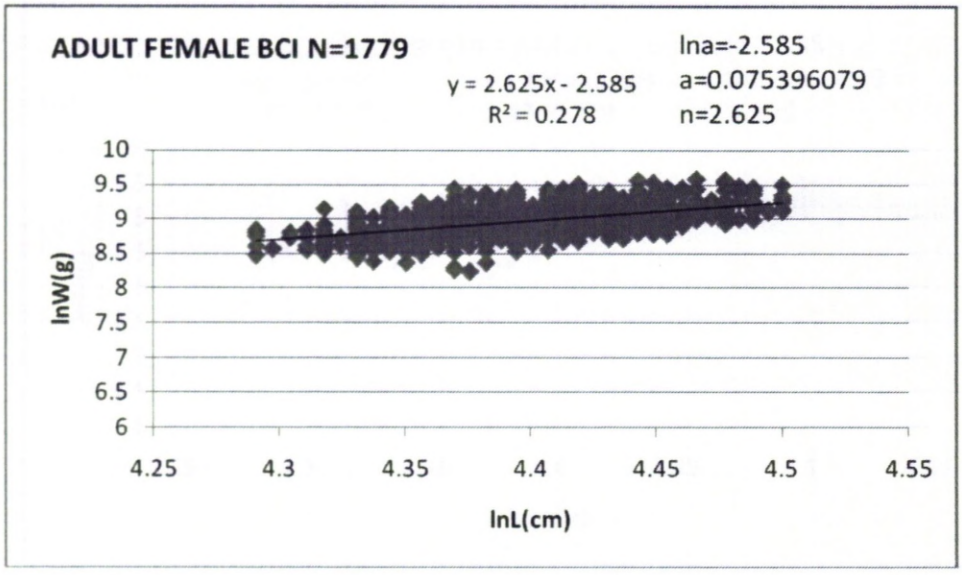

Figure 3. Calculation of a BCI for adult female badgers, captured at Woodchester Park 1997-2009.

Resulting equations for BCI calculations:

Cubs	BCI = W (g) / 0.00128114637 * L (cm) ^3.55
Adult males	BCI = W (g) / 0.028955358 * L (cm) ^ 2.848
Adult females	BCI = W (g) / 0.075396079 * L (cm) ^ 2.625

This approach has been successfully employed in previous studies for the calculation of body condition scores for otters (Kruuk *et al.* 1987), and was subsequently applied to badgers (Woodroffe & Macdonald, 1995). Mean monthly condition index (BCI) values were used to investigate changes in the body condition of cubs, adult male and adult female badgers respectively. In both adult sexes body condition peaked over the late autumn and early winter months and declined to its lowest point during mid to late summer (Figure 4). However, the pattern of BCI variation appeared different for cubs, with cubs in relatively better condition in the summer presumably pre-weaning and showing a more marked loss in body condition into the autumn and early winter consistent with post-weaning and having to forage more independently.

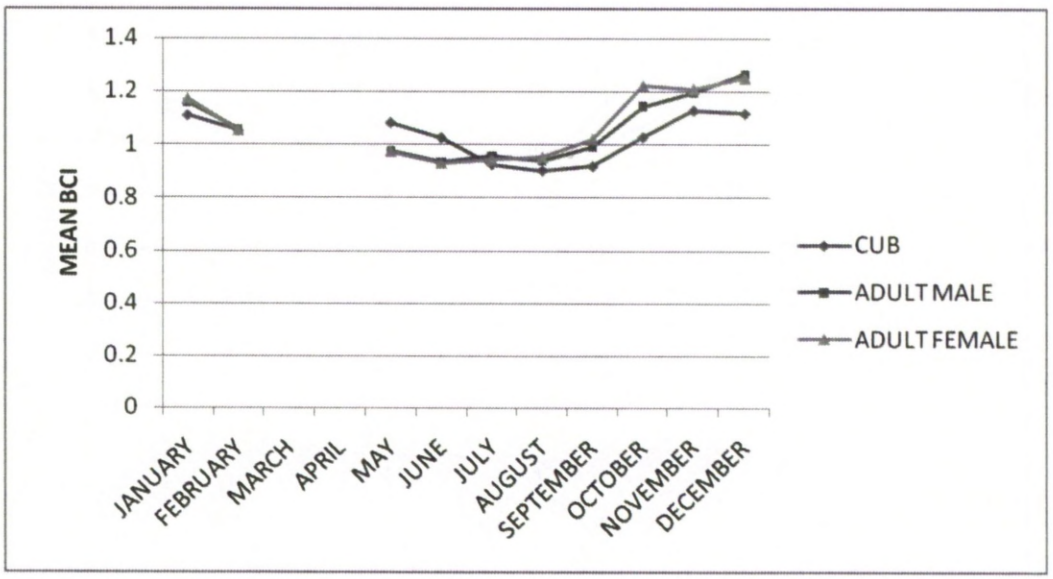

Figure 4. The trend in mean monthly body condition index (BCI) scores for cubs, adult males and adult females respectively (1997 - 2009).

Standardisation of body condition index

To normalise the BCI value, and control for seasonal, sex and age variations in body condition, all values were standardised using the equation below:

BCI (adj) = BCI (value) – BCI (mean for age, season and sex) / SD (for age, season, sex)

Age and season categories were as shown in Tables 1 and 2. Standardisation of all values resulted in a BCI variable that centred around zero and was considered to be independent of season, and badger age or sex.

Table 1. Categorisation of badger age for standardisation of BCI

Age in years	Category
0	Cub
1 and 2	Juvenile
3,4,5,6	Adult
>6	OAP

Table 2. Categorisation of seasons for standardisation of BCI

Month	Season
December/January/February	Winter
March/April/May/June/July/August	Summer
September/October/November	Autumn