Variation in the immune response of badgers and the diagnosis of bovine tuberculosis

Submitted by Laura Jane Waring to the University of Exeter as a thesis for the degree of Masters by Research in Biological Sciences, January 2016

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ABSTRACT

Diagnosing diseases in wildlife is challenging, the availability of diagnostic tests that perform at the same level of sensitivity and specificity in wild animals as their human and livestock counterparts is limited. In the last few decades increased awareness of the role of wild animals in the maintenance and spread of important zoonotic infections means that research has been directed into development of specific diagnostic tests aimed at wildlife that can be used for research, surveillance and wildlife control programmes.

Wild animals are subject to a wide range of genetic and environmental factors many of which may influence their immune response. These additional pressures may affect the immune system in different ways and within a group of animals; individuals will vary. The first aim of this dissertation was to investigate variation in the cell-mediated immune (CMI) response of free-living badgers to a range of intrinsic factors. Results revealed factors that may suppress the non-specific immune response such as old age, presence of wounds and larger social group sizes and those that amplify the specific *M. bovis* response such as age, presence of wounds and bTB exposure. Results also showed complex interactions between body condition with age, sex and season which influenced both the specific and non-specific CMI response. Second, to aid in disease surveillance and management, we developed and evaluated a technique to collect and test blood from unanaesthetised badgers in the field. This study successfully demonstrated proof of concept where a method of capillary blood collection used in the field enabled the operator to carry out a rapid diagnostic

test for the presence of *Mycobacterium bovis* infection. In addition we have shown that the accuracy of the DPP VetTB test carried out in the controlled conditions of the laboratory cannot be replicated when testing under field conditions suggesting that stochastic conditions in the field may affect TB diagnostic outcomes.

My findings illustrate the importance of understanding variation in test performance, arising from intrinsic and extrinsic factors in diagnosing and managing bovine tuberculosis in badgers, and other diseases of highly variable wild hosts.

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CHAPTER 1 Introduction to Wildlife Diseases

Disease can, in its broadest sense, be regarded as anything that causes impairment of the normal function of a living organism (Delahay *et al.*, 2009). Infectious disease refers to the ability of parasites or pathogens to go through the process of entering another organism, replicating and causing harm (Ross, 1983). These parasites/pathogens have been divided into two broad categories, the macroparasites and microparasites, based, to some extent on their size, but primarily on their mode of replication, population biology and the nature of their interaction with the host (Anderson, 1982). Macroparasites include helminths and arthropods and are more commonly associated with more chronic infections. Macroparasites are typically larger than microparasites and immunity is of short duration in heavily infected hosts resulting in infections that are persistent and where reinfection is common (Wobeser, 2002). Microparasites include bacteria, viruses, fungi and protozoa and are frequently associated with acute disease. Microparasites have a short generation time and usually multiply rapidly within the host; hosts that recover from infection usually have long-lived immunity that protects against re-infection (Delahay *et al.*, 2009).

In recent decades infectious pathogens found in wild animal species have become increasingly important in infectious disease research with particular interest in diseases that have a substantial impact on human health, agricultural production, biodiversity, animal welfare and the economy (Bengis *et al.*, 2004). Some pathogens cause significant disease in their wildlife host and can have an

effect on their behaviour, fecundity, growth or metabolic requirements and in some cases cause death, whilst others may not induce overt illness in the host but instead lead to the host becoming a reservoir for the pathogen (Williams *et al*., 2002), the importance of understanding this role as reservoir is becoming more widely appreciated (Simpson, 2002).

Human activities including concurrent human and domestic animal population expansion and encroachment into wildlife habitat, international movement of livestock and modern agricultural practices, introduction of domestic and wild animals into new habitats (Daszak *et al*., 2001) along with environmental change are resulting in new infectious disease dynamics and patterns favourable to pathogens spreading both geographically and between species as well as new opportunities for increasing genetic variability (Howard and Fletcher, 2012). The increasing emergence of disease in wildlife populations may be a natural phenomenon or anthropogenically driven and in some cases there may be severe consequences for wildlife populations. Examples of such cases include the Chytrid fungus (*Batrachochytrium dendrobatidis*) which has been linked to dramatic population declines and extinction in some species of amphibian over several continents (Stuart *et al*., 2004), white-nose syndrome associated with high mortality in bat species in the Northern US (Blehert *et al*., 2009) and Devil facial tumour disease causing a decline in Tasmanian devil (*Sarcophilus harrisii*) populations (McCallum, 2008).

Wildlife also plays a major role in disease transmission and this should be taken into consideration when addressing certain diseases in domestic livestock and humans. Infectious diseases that can be transmitted from animals to humans

are referred to as zoonotic. A study by Jones *et al*. (2008) found that 60% of emerging infectious disease (EID) events in humans were caused by zoonotic pathogens, of which 72% were from a wildlife origin. Humans can be infected by diseases carried by wildlife both directly and indirectly through vectors such as ticks and mosquitoes. In some wildlife diseases, transmission of the pathogen to humans is a rare event but once it has occurred human to human transmission maintains infection within the population. Examples of such cases include human immunodeficiency virus (HIV), Ebola, influenza A and severe acute respiratory syndrome (SARS) (Bengis *et al*., 2004). Other infectious pathogens may transmit to humans from animals but are not generally transmitted from person to person. For example, the arenavirus haemorrhagic fevers, Hantavirus pulmonary syndrome and Nipah virus (Morens *et al*., 2004).

Diseases found in both wild and domestic animals have two major implications, economic impact due to the effect of disease on the domestic species itself and the risk the diseases pose to human health. The 2001 foot and mouth disease outbreak is said to have cost the UK around £3.1 billion in losses to agriculture and the food chain (Thompson *et al*., 2002) and the bluetongue serotype 8 epidemics of 2006 and 2007 in the Netherlands had an estimated net cost of 32.4 million Euros in 2006 and between 164 and 175 million Euros in 2007 (Velthuis *et al*., 2010).

Bovine tuberculosis (bTB) is among the most important zoonotic diseases at the livestock-wildlife interface and has a significant impact on human health and the economy (Krebs, 1997; Michel *et al*., 2010). *Mycobacterium bovis* is the causative agent of bTB. *M. bovis* is of public health concern especially in

developing countries due to its zoonotic potential (Cosivi *et al.*, 1998; Theon, 2006). *M. bovis* infection in badgers (*Meles meles*) in the UK (Clifton-Hadley *et al.*, 1993) and Republic of Ireland (Gormley and Collins, 2000) is a major source of infection in cattle. *M. bovis* infection causes similar problems in other wildlife species around the world including the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Coleman and Cooke, 2001), white-tailed deer (*Odocoileus virginianus*) in the United States (Schmitt *et al.*, 1997), European wild boar (*Sus scrofa*) in Spain (Naranjo *et* al., 2008) and African buffalo (*Syncerus caffer*) in Africa (Vos *et al.*, 2001).

With the increasing knowledge that wildlife species can act as a reservoir of diseases transmissible to humans and domestic animals there is a growing interest in developing methods to control transmission. Control measures have included setting up barriers, hygienic measures, habitat management, vector control, treatments, fertility control, culling, vaccination and test-and-slaughter policies (Wobeser, 2002; Artois, 2003; Killian *et al.*, 2007; Bengis *et al.*, 2002). In some cases some of these control methods may be impractical or difficult to carry out and culturally or morally unacceptable in wildlife populations (Bengis, 2002).

In the case of bTB in the UK various control options have been considered to try and prevent spread between badgers and livestock. From 1973 until 1997 (Krebs *et al.*, 1997), and again since 2011 (DEFRA, 2011) the nonselective culling of badgers has formed part of bTB control policy in the UK. However this control strategy has been unpopular with the British public. In consideration of animal welfare and the unpopularity of culling, vaccination may be an alternative

or additional option. Badgers could be vaccinated against *M. bovis* infection with the aim to reduce spread amongst badgers and to other animals (Buddle *et al.*, 2000; Chambers *et al.*, 2011; Carter *et al.*, 2012) and with access to a suitable diagnostic test that could be used at trap-side, a test vaccinate/remove (TVR) programme might be a more attractive option and possibly more publicly acceptable (Bielby *et al.*, 2014).

Testing for disease in wildlife is usually more difficult than in domestic animals. Wild animals are free-living and therefore not constrained by barriers and can range over large areas (Mörner *et al.*, 2002). This makes them more difficult to access compared to domestic stock so identifying signs of disease and diagnosis can be more challenging and expensive. For the majority of diseases found in both wildlife and domestic animals, assessments of the sensitivity and specificity of a diagnostic test have been based on the domestic species. Sensitivity and specificity of a test can vary considerably due to differences in pathogenic strains and serovars, host serological responses, and exposure to organisms of similar antigenic structure that produce cross-reacting antibodies (Gardner *et at.*, 1996). In addition individual wild animals are inherently more variable than livestock, and to some extent humans, in that they experience a wider range of conditions. Thus diagnosis in wild animals interacts markedly with between-individual variation in state/condition. With this in mind the test cannot be assumed to have the same accuracy in its wildlife counterpart (Bengis *et al.*, 2002). The availability of diagnostic assays is a pivotal issue when managing bTB in wildlife. Many of the tests available have been developed for use in domestic animals and humans and therefore the sensitivity and specificity are unlikely to be the same.

Recognition of the role that some wildlife species have as a maintenance host that could cause a spill-over of disease to other species has led to more focused studies with development of *M. bovis* diagnostic tests in a variety of wildlife species (Chambers, 2013; Maas *et al*. 2013). Three diagnostic tests are used routinely in studies of badgers and include culture (Gallagher and Horwill, 1977), StatPak serological assay (Chambers *et al*. 2008) (recently discontinued by the manufacturer and superseded by the dual path platform VetTB assay, Chembio, Medford, NY, USA, currently under evaluation in badgers) and the interferon-gamma enzyme linked immunosorbent assay (IFN_{γ} ELISA) (Dalley *et al*., 2008). Bacterial culture of a range of clinical samples including faeces, urine, tracheal aspirate and bite wounds requires the animal to be anaesthetised for collection. Culture has a low sensitivity of around 8% (Drewe *et al*., 2010) and can take up to 8 weeks for results with specialist laboratory equipment required. The StatPak and VetTB assays require a blood sample that is taken under anaesthetic however due to the small quantity required for the VetTB assay (10 µl) it has the potential as a trap-side test. Results are rapid (20 minutes from application) and no specialised laboratory equipment is required. The StatPak assay has only moderate sensitivity of 50.4% (Drewe *et al*., 2010) and the sensitivity of the VetTB is yet to be determined. The IFN_{γ} ELISA measures the cell-mediated immune response, based on measures of IFN_{γ} released in response to specific mycobacterial antigens PPD B and PPD A. This more sensitive assay (80.9%) requires blood taken under anaesthesia to be processed within 7 hours (Dalley *et al*., 2008). Results are available in 48hrs though the test requires specialist laboratory equipment. The IFN_{γ} ELISA

has the added advantage of incorporating assay of the response to a non-specific immune stimulant pokeweed mitogen (PWM) as a positive control.

In Chapter 2 of this thesis, I investigate variation among a wild population in their IFN_{γ} responses to both the specific (PPD A and PPD B) and non-specific (PWM) immune stimulants. My aim is to investigate the influence of a range of intrinsic factors on the magnitude of badgers' cell-mediated immune responses. Monitoring of *M. bovis* infection in live badgers is currently carried out by taking various samples from badgers that have been trapped and anaesthetised. For a selective control programme to be feasible, a more efficient way to test badgers would be required. In Chapter 3 I investigate a blood collection technique on unanaesthetised badgers at trap-side where wild badgers were kept in traps for a relatively short period, during which a small blood sample was taken and a serological test carried out. My aim is to develop a repeatable method for capillary blood collection which can be used in the field to test for the presence of *Mycobacterium bovis* infection in badgers.

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CHAPTER 2 Variation in the interferon-gamma response of wild badgers *Meles meles*

2.1 Abstract

In the UK and Ireland, European badgers (*Meles meles*) are an important wildlife reservoir of *Mycobacterium bovis*, the causative agent of bovine tuberculosis. An important component for development of strategies to control *M.bovis* in badgers involves accurate diagnosis of infection in the live animal for which there are various diagnostic tests available. There are, however, limitations with these tests especially when used in isolation from other clinical information. Host-pathogen interaction in tuberculosis infections is a complex process and the cell-mediated immune response has been observed in other species as the dominant response at early stages of anti-microbial immunity. The gamma interferon (IFN_{γ}) enzyme linked immunoassay (ELISA) has been developed to detect the cytokine IFN_{γ} an important component of the cell-mediated immune response. IFN_{γ} production is critical in determining the effectiveness of the immune response to intracellular pathogens such as *M. bovis*. Various intrinsic factors may affect the level of IFN_{γ} produced to both specific and non-specific immune stimulants. This study investigates a number of intrinsic factors to determine if they have an influence on the cell-mediated immune response of badgers. The results show that age, season, year, body condition, wounds, exposure to *Mycobacterium bovis* (bTB exposure), group size and various interactions with body condition including age, sex and season significantly influence the immune response of badgers either specifically in

response to PPD A and PPD B tuberculin or non-specifically to PWM, or both. This study has revealed factors that may suppress the non-specific immune response such as old age, presence of wounds and larger social group sizes and those that augment the specific *M. bovis* response such as age, presence of wounds and bTB exposure. Year and season appear to be common factors for both with a drop in IFN_{γ} levels over the study period with the highest levels apparent during winter. These results provide insights into potential determinants of susceptibility to infection and detection of infection in badgers, through suppression and enhancement of non-specific and specific immune responses.

Keywords: *Mycobacterium bovis;* gamma interferon; intrinsic factors; cell-mediated immune response

2.2 Introduction

Bovine tuberculosis (bTB) is an infectious, zoonotic disease that causes a significant economic burden and financial loss to taxpayers and the livestock industry. Bovine TB is endemic in many countries (Schiller *et al.*, 2010) and since the early 1990s the UK has seen a rapid increase in the incidence of bTB in cattle (Godfray *et al.*, 2013). The causative agent of this disease is the intracellular acid-fast bacterium *Mycobacterium bovis.* It was first suggested that the European badger (*Meles meles*) was a source of infection for cattle in the 1970s (Muirhead *et al.*, 1974) and badgers are still the principal wildlife species implicated in bTB epidemiology in the UK and Ireland.

The host-pathogen interaction in tuberculosis infections is a complex process (Boom, 1996; Pollock *et al*., 2001). The cell-mediated immune response has been observed in other species as the dominant immune response at early stages of anti-microbial immunity (Griffin *et al*., 1991; Gutierrez *et al*., 1998; Ritacco *et al*., 1991). A study by Cassidy *et al* (2001) has shown that in cattle T-cells are among the first cells involved at the site of infection with *M. bovis*. Cattle infected experimentally with *M.* bovis have shown that the CD4+ memory T-cells appear to be the dominant cells producing interferon gamma (IFN_γ) with CD8+ memory T-cells also producing IFN_γ (Hope *et al*., 2000). IFN_γ production is critical in determining the effectiveness of the immune response to intracellular pathogens (Frucht *et al*., 2001) and has been shown to modulate the immune response to a variety of intracellular pathogens, including *M. tuberculosis* (Flynn *et al*., 1993). *M. bovis* can survive within the macrophage (Aldwell *et al*., 2001) and IFN_γ is thought to be critically involved in the immune response due to its major role in macrophage activation, inducing the release of mycobactericidal nitric oxide (Bonecini-Almeida *et al*., 1998; Flynn *et al*., 1993). Stimulation of the memory T-cell response with mycobacterial antigens therefore provides a means to detect *M. bovis* infection (Pollock *et al*., 1996) as IFN_γ can be released in vitro and then quantified by means of an enzyme linked immunosorbant assay (ELISA).

Many wildlife species are exposed to environmental mycobacteria (Stainsby *et al*., 1989). Balseiro *et al*. (2002) provided evidence of environmental mycobacteria *Mycobacterium avium* complex (MAC) in badgers in the UK which they attributed to badgers' lifestyle of digging, living underground and broad diet, including earthworms and other invertebrates (Neal and Cheeseman,

1996). Infection by environmental mycobacteria can result in high levels of IFN_{γ} production *in vitro* from lymphocytes stimulated with bovine tuberculin, due to the presence of antigens common to both mycobacterial strains. In non-infected animals, potential cross-reactions can lead to false-positive diagnoses. Where this occurs, comparison with responses to avian tuberculin is often used to facilitate discrimination between cattle infected with *M. bovis* and those exposed to environmental strains (Pollock *et al*., 2005). It has also been shown in experiments with guinea pigs that prior exposure to environmental mycobacteria can have a modulatory effect on the protective efficacy of the Bacille Calmette-Guérin (BCG) vaccine due to shared mycobacterial antigens present (Kamala *et al*., 1996). Exposure to these environmental mycobacteria may therefore also contribute to the immune response by acting as immunomodulators due to common/cross-reacting antigens between *M. bovis* and MAC species.

An important component of strategies to control *M. bovis* in cattle and badgers involves accurate diagnosis of infection in the live animal. There are various diagnostic tests available, with differing levels of performance in identifying infected badgers. Although culture of tissues obtained *post mortem* is considered to be a 'gold standard', culture has relatively low sensitivity when applied to clinical samples due partly to the intermittent excretion of detectable levels of bacteria (Chambers *et al*., 2002; Gavier-Widen *et al*., 2001; Neill *et al*., 1988). Alternative *in vitro* diagnostic tests include serological assays, such as the Brock StatPak assay (Chambers *et al*., 2008) drawbacks of which include cross-reactivity with other environmental bacteria (Stainsby *et al*., 1993) and potential delays in seroconversion (Hanna and Neill, 1992).

Diagnostic tests that detect early stages of infection (Pollock and Neill, 2002; Vordermeier *et al*., 2002) are based on measurement of the cell-mediated immune response and have been found to be more sensitive (Wood, *et al., 1992*). The interferon gamma enzyme-linked immunosorbant assay (IFN_{γ} ELISA) is one such test. In this test, sampled lymphocytes are stimulated overnight with bovine tuberculin, which is a purified protein derivative of *M. bovis* (PPD B) and the subsequent plasma harvested from this is tested for the presence of gamma interferon released in response to *M. bovis* infection by the IFN_{γ} ELISA (Dalley *et al*., 2008). PPD B tuberculin is prepared by precipitation of heat-killed cultures of *M. bovis* and contains a poorly defined mixture of antigens (Paterson, 1948). Prior exposure to environmental mycobacteria may cause a cross-reaction, as demonstrated in studies of cattle (Aagaard *et al*., 2010; Schiller *et al*., 2010). Addition of an avian tuberculin, purified protein derivative (PPD A), used alongside PPD B reduces the likelihood of false positive results due to environmental mycobacteria, as a positive result is based on the optical density of the PPD B response minus the optical density of the PPD A response.

In badgers *M. bovis* may persist in a clinically latent state, potentially creating a population of 'silent' *M. bovis*, which can reactivate later in life and represent a major source of disease (Gallagher and Clifton-Hadley, 2000). Infection in badgers is complex; some animals appear capable of containing the infection without showing clinical or pathological signs, though they intermittently excrete bacilli. A study by Gallagher *et al* (1998) observed pale/partially stained acid fast bacilli, typical of dead/inactive mycobacteria, in microscopic lesions in badgers considered to be not visibly lesioned (NVL) at *post-mortem*. Given that

some lesions were also free of bacilli, the author suggested this may indicate the resolution of infection with maintenance of a memory T-cell response.

Mitogens such as phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) have been used in many studies to investigate the ability of lymphocytes to proliferate, and as an indicator of cell viability (Lesellier *et al.*, 2008). Such nonspecific stimulants of lymphocytes reveal the more generalised immune function and response of T-cells to a wide range of pathogens and not just exposure to antigen-specific ones (Jackson *et al.*, 2011).

Across most of the UK, badgers are group living and aerosol exposure to *M. bovis* is considered to be the most frequent route of infection, with the lungs and the associated lymph nodes as the primary site of infection (Gallagher *et al.*, 1976). Excretion of *M. bovis* may occur in sputum, faeces, urine, and wound exudate (Gallagher and Clifton-Hadley, 2000). Badgers have a fossorial lifestyle, complex social group structure and exhibit territorial aggression, all of which may influence transmission of infection. Although various intrinsic factors may be expected to affect the magnitude and character of cell-mediated immune responses in infected badgers, detailed studies are scarce. The aim of this study is to investigate the potential effects of intrinsic factors such as sex, age, bite wounds, body condition, social group size, season, year and variation in exposure to *M. bovis* (bTB exposure), on the magnitude of multiple IFN_{γ} responses in badgers (i.e. antigen specific PPD A and PPD B tuberculin and the nonspecific immune stimulant PWM). Knowledge of individual variation in responses has a clear bearing on ecological immunology and is also informative with respect to disease diagnostics in wildlife populations.

2.3 Materials and Methods

Study area and badger sample collection

Life-history data including diagnostic test results were collected from an intensively studied, high-density, population of wild badgers, which is naturally infected with *M. bovis.* The study population was located within a study area of approximately 7 km^2 at Woodchester Park, Gloucestershire, UK (Rogers *et al.*, 1997; Delahay *et al.*, 2000). This badger population has been the subject of a capture-mark-recapture study for over 30 years. Data included in the present study originated from badgers captured from July 2006, when the IFN_{γ} ELISA was first introduced, until July 2013. During this period, consistent testing protocols were used with an equal distribution of seasonal trapping events. Details from 528 individuals in 27 social groups were recorded during this period, with social group structure and social group membership determined by capture histories and an annual bait-marking exercise to map territories (Delahay *et al.*, 2000).

Trapping procedures including the handling and sampling of badgers have been described elsewhere (Cheeseman *et al.*, 1987; Delahay *et al.*, 2013). Briefly, traps were set near active setts/runs associated with each social group, four times per year. Trapped animals were anaesthetised before weight (kg), body length (cm), and evidence of bite wounds were recorded. Blood samples were collected for serology (Chambers *et al.*, 2008) and IFN_{γ} ELISA (Dalley *et al.*, 2008) and clinical samples were taken including faeces, urine, tracheal and oesophageal aspirate and bite wound swabs for bacteriological culture of *M.bovis* (Gallagher and Horwill, 1977).

Sample Preparation and IFN$_{\gamma}$ testing

The IFN$_{\gamma}$ test is carried out in two stages, whole-blood stimulation and IFN$_{\gamma}$ ELISA (Dalley *et al.*, 2008). The initial step requires the presence of live white blood cells and therefore whole blood was processed within 7 hours of sampling (Coad *et al.*, 2007; Waters *et al.*, 2007). Heparinised whole-blood was cultured in the presence of both PPDA and PPDB antigens at 30 μg/ml, PWM at a concentration of 5 μg/ml and a no antigen control (Nil). After 16-24hrs incubation at 37°C, the plasma was separated from these four cultures and 250μl was stored in a 96 well plate. Plasma was then frozen at -80°C prior to IFN$_{\gamma}$ ELISA testing. Stimulated plasma was tested in duplicate (100 μl/well) by a monoclonal sandwich ELISA (IFN$_{\gamma}$ ELISA) with the end product providing a measurement of IFN$_{\gamma}$ produced in the plasma as an optical density (OD) measured at 450nm (OD_{450}) (Dalley *et al.*, 2008). Samples were tested in duplicate and therefore two OD readings were available for each antigen and the nil control.

Body condition

In order to determine the body condition of each individual badger the scaled mass index (SMI) was calculated following the method by Peig and Green (2009), using the following equation:

$$\widehat{M}_i = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$$

Where M_i is body mass and L_i the linear body measurement of individual i, b_{SMA} is the scaling exponent estimated by the standardised major axis (SMA)

regression of M on L; L_0 is the mean value of the study population; and $\widehat{M}_i$ is the predicted body mass for individual i when the linear body measure is standardised to L_0. In this study, the scaling coefficients were calculated for all badgers using data from 2105 capture events obtained from the Woodchester Park database between July 2006 and July 2013. The mass was measured in kilograms and body length in cm, $b_{SMA} = 3.81$ and $L_0 = 80.35$ cm with the final calculated SMI measured in kg.

Group size and exposure

Badger social group size was calculated by counting the total number of individual animals trapped, within each social group, during the 11 months prior to each trapping event. The likely exposure of an individual badger to *M. bovis* within its social group was estimated by ascribing a disease status to each badger based on diagnostic test results from serology and culture, but not IFN_γ. If an animal was negative by both culture and serology tests and had never previously tested positive at any point on either assay, it was deemed negative and given a score of zero. If there had ever been a positive serology result then the badger was classed as seropositive from the first positive test onwards (irrespective of any subsequent negative tests) and given a score of one. If an animal tested positive for culture of *M. bovis* it was deemed an excretor and give a score of two from that point onwards (irrespective of any subsequent negative cultures). The scores of animals resident in each group were then added together (excluding the subject individual) to produce an overall disease exposure score for each individual for each capture event.

Statistical analyses

To test the association between duplicate IFN_{γ} OD results for each sample, Pearson's product moment correlation was calculated for each of the duplicate responses for each of three test outcomes (PPDA, PPDB and PWM). A highly significant correlation ($p<0.001$) was observed for all three and so a mean of the duplicates was calculated for use in the data analyses.

To investigate relationships amongst the three measures of immune response, Pearson's product moment correlations were calculated and a highly significant correlation ($p<0.001$) was observed between all variables. A principal component analysis of variation in the responses to PPD A, PPD B and PWM was then carried out. This was to create a variable (PC1) that captured the correlated elements of variation in the overall magnitude of the three IFN_{γ} responses and a second, orthogonal component (PC2), which may indicate a more specific component to variation in response size.

To investigate which factors influenced the magnitude of badger immune responses based on cytokine IFN_{γ} release, after stimulation with PPD A, PPD B and PWM, a series of generalised linear mixed models (GLMM) were fitted. There were three types of response variable. First, the mean OD_{450} values observed from PPD A, PPD B and PWM were taken as specific responses and were log_{10} transformed to meet normality assumptions (Quinn and Keough, 2002). Second, the first principal component (PC1) was used to reflect between-individual variation in the overall magnitude of IFN_{γ} production while the second principal component (PC2) was used as a measure of variation in the antigen-specific response. Third, due to the likely cross-reactivity of

environmental mycobacteria with the PPD B tuberculin, an additional analysis was carried out on PPD B OD_{450} minus PPD A OD_{450} (B-A). This last response (B-A) corresponds to the measure used routinely in bTB diagnostics in badgers (Dalley *et al.*, 2008). Fixed effects in all models remained the same for all analyses and included individual age (two categories: cubs=≤1 year old and adults=>1 year old), sex, season (four categories: spring=March-May, summer=June-August, autumn=September-November, winter=December-February), year of capture (2006-2013), group size (GS), group bTB exposure score (bTB exp), wounds present (three categories: Absent, Fresh, Healed) and Scaled Mass Index. Potential two-way interactions included in all the models were: age*sex, age*season, age*wounds present, age*SMI, age*group size, sex*season, sex*wounds present, sex*SMI, sex*group size, season*SMI. Social group and individual identity (ID) were fitted as random effects in models to account for repeated measures of individuals and variation among social groups.

To improve interpretation and place all input variables on a common scale, variables were standardised to a mean of zero and a standard deviation of two prior to analyses (Gelman, 2008). Model selection was restricted to a top model set based on a cut-off criterion of **Δ**AICc = 2. The average model coefficients were then calculated based on this top model set using the natural average method (Burnham and Anderson, 2002). Explanatory variables were deemed to have an effect on the magnitude of immune response if the coefficient confidence intervals did not span zero (Grueber, 2011). The AICc value and Delta value (difference in AICc score from the best candidate model) represents the fit of models and the weight value, interpreted as the probability of a given

model being the best at explaining the data. All GLMM analyses were carried out using package ‘lme4’ in the statistical package R 3.0.1 (cran.r-project.org) and models containing different combinations of fixed effects were compared using the R 3.0.1 package ‘MuMIn’.

2.4 Results

A total of 528 individuals (238 males, 290 females) were represented in the 2105 capture events, of which 1347 were of adults and 758 of cubs. Healed or fresh wounds were recorded on 24 % of capture events for adult badgers and 3.4% for cubs. The mean prevalence of infection (based on results from routine diagnostic tests) and group size for the 27 social groups observed in this study are summarised in Table 2.1.

The first two principal components accounted for 76% of the total variation in responses to PPD A, PPD B and PWM. PC1 explained 48% of variance and showed strong negative correlations with the magnitude of all three antigens (PPD A: -0.599; PPD B: -0.522; PWM: -0.607), and so reflected between-individual variation in the overall magnitude of IFN_{γ} production. To ease subsequent interpretation, PC1 scores were inverted (0-PC1) so that a greater value indicated a larger response. PC2 explained 28% of variance and showed a strong positive correlation (0.851) with PPD B and a negative correlation with both PPD A (-0.415) and PWM (-0.322) thus distinguishing variation in IFN_{γ} production among antigens, likely identifying *M. bovis* infected individuals and an elevated specific immune response.

The top models for each response variable varied slightly in terms of the explanatory variables and interactions that were retained (see Table 2.3, Appendix I). GLMMs indicated that variation in responses to PWM, PPD A, PPD B, B-A, PC1 and PC2 were significantly associated with various intrinsic factors (see Table 2.2). Confidence intervals and relative importance quoted for significant results can be found in Table 2.4 (Appendix II).

Table 2.1. Mean annual abundance of bTB test positive badgers including range (in brackets), and group size for each social group for the study period (2006-2013). Abundance is the mean number of animals per year that tested positive to any of the three routine diagnostic tests (IFN_{γ}, StatPak and culture) for the study period (2006-2013).

Social Group	**Abundance of test positive badgers**	**Group Size**	**Social Group**	**Abundance of test positive badgers**	**Group Size**
Arthurs	4.50 (0-12)	10.08 (0-18)	Old Oak	3 (0-8)	8.55 (1-13)
Beech	4.38 (0-10)	11.87 (0-17)	Park Mill	6.63 (0-16)	11.22 (1-15)
Breakheart	1.75 (0-6)	4.04 (0-7)	Peglars	0.88 (0-4)	5.19 (2-7)
Cedar	5.25 (1-10)	8.42 (2-15)	Scotland Bank	0.88 (0-4)	3.40 (1-4)
Cole Park	0.25 (0-1)	1.00 (0-2)	Septic Tank	6 (1-9)	4.10 (1-7)
Colliers Wood	1.88 (0-6)	3.67 (0-6)	Top Sett	4.75 (0-13)	6.99 (3-13)
Hedge	0.38 (0-3)	12.39 (5-17)	Trackside	1.75 (0-5)	4.71 (0-7)
Honeywell	3.63 (0-12)	10.05 (4-16)	West	3.88 (0-8)	8.85 (5-15)
Inchbrook	1.75 (0-4)	6.77 (3-10)	Windsor Edge	8.75 (3-18)	8.54 (3-13)
Jacks Mirey	5.25 (2-8)	6.62 (1-12)	Wood Farm	0.38 (0-2)	4.88 (2-8)
Junction	1 (0-4)	9.97 (2-16)	Woodrush	2 (0-4)	3.38 (0-6)
Kennel	2.13 (0-4)	7.31 (0-10)	Wych Elm	2.38 (0-7)	7.60 (3-11)
Larch	10.25 (2-19)	11.58 (7-16)	Yew	3.50 (1-6)	11.79 (4-19)
Nettle	4.75 (0-11)	5.80 (3-11)			

Table 2.2. Factors affecting variation in the magnitude of IFN_{γ} responses to multiple antigens in badgers. Values are the coefficients of standardised predictor variables from the averaged set of top models. Significant values, where confidence intervals do not overlap 0 are shown in bold. Missing values are where that factor did not appear in the top model sets for that response. PPD A is the response to stimulation to avian tuberculin, PPD B is bovine tuberculin, PWM is pokeweed mitogen, PC1 is the first component of a principal component analysis of the first three variables and reflects overall magnitude of IFN_{γ} responses while PC2 is positively associated with a specific response to PPD B relative to PPD A and PWM. B-A is PPD B – PPD A and is the main metric used in bovine tuberculosis diagnosis in badgers. The reference levels for each factor are: sex = female, age = adults, season = spring, wounds = absent. For confidence intervals and relative importance see Table 2.4 (Appendix II).

Variables	**PPD A**	**PPD B**	**PWM**	**PC1**	**PC2**	**B-A**
Sex: Male	0.012	-0.012	-0.041	-0.046	-0.083	-0.004
Age: Cubs	-0.036	**-0.058**	**0.113**	0.142	**-0.153**	-0.002
Season:Summer	-0.020	0.008	**-0.071**	-0.127	**0.106**	
Season:Autumn	-0.018	0.019	**-0.122**	**-0.239**	**0.146**	
Season:Winter	**0.066**	**0.103**	**0.095**	**0.467**	-0.025	
Year	**-0.189**	**-0.198**	**-0.318**	**-1.229**	**0.333**	**-0.004**
Scaled Mass Index	-0.001	-0.035	**0.082**	0.091	0.016	0.000
Wounds: Fresh		**0.067**	**-0.076**		**0.196**	**0.007**
Wounds: Healed		0.033	-0.037		0.055	0.001
Group bTB exposure score	0.009	**0.124**	0.025	**0.233**	**0.239**	**0.010**
Group size	**0.016**	-0.028	**-0.051**	**-0.150**		-0.002
Age x Sex	-0.029		-0.015		0.147	0.005
Age x Season:Winter	-0.003			-0.205	-0.088	
Age x Season:Autumn	0.050			0.167	-0.096	
Age x Season:Summer	-0.016			-0.161	0.088	
Age x Wounds:Fresh			-0.115			
Age x Wounds:Healed			0.078			
Age x Group Size	-0.008					
Sex x Season:Winter			0.100			
Sex x Season:Autumn			0.076			
Sex x Season:Summer			0.032			
Sex x Wounds:Fresh			-0.106			-0.007
Sex x Wounds:Healed			0.008			0.004
SMI x Age: Cubs		**0.054**	0.029	0.088		
SMI x Sex: Male	**0.034**	0.033	-0.061			
Sex x Group Size		-0.040	-0.025	-0.123		
SMI x Season:Winter	-0.051	-0.051	**-0.196**	**-0.478**		
SMI x Season:Autumn	0.019	0.050	**-0.091**	-0.028		
SMI x Season:Summer	-0.006	0.028	-0.060	-0.099		

Sex

There was no significant effect of sex as a main effect on the magnitude of IFN_γ for any of the response variables.

Age

When compared to adults, cubs produced significantly lower IFN_γ responses to PPD B and PC2 but higher responses to the more general stimulus of PWM (Figure 2.1).

Season

All response variables showed significant variation among seasons (Figure 2.2), apart from the bTB diagnostic measure B-A where season did not appear in any of the thirteen top models. IFN_γ production was significantly greater in winter for PPD A, PPD B, PWM and especially for PC1. Significantly reduced IFN_γ production was noted for PWM and PC1 in autumn and for PWM in summer. PC2 showed opposite trends where IFN_γ production was significantly greater in autumn and summer.

Year

Year of capture and testing had a significant effect on all the response variables analysed. A marked decline from 2006-2013 was apparent for PPD A, PPD B, PWM, and PC1. However, a positive trend with time was apparent for PC2 (Figure 2.3).

Body condition

The effects of body condition (Scaled Mass Index SMI) on IFN_{γ} production interacted significantly with sex, age and season (Figure 2.4). For PPD A, the effect of body condition on IFN_{γ} response was significantly greater in male badgers than females. For PPD B, the effect of body condition on IFN_{γ} response was significantly greater in cubs than in adult badgers. The effects of body condition also varied among the seasons. For PWM, IFN_{γ} levels were negatively related to condition in winter but positively related to condition in spring. For PC1, a similar pattern occurs where IFN_{γ} production is negatively related to condition in winter but positively correlated during spring, summer and autumn. After addressing significant interactions, there remained a positive effect of condition on IFN_{γ} response to PWM (Figure 2.4e).

Wounds

Badgers with fresh wounds produced a significantly higher response to PPD B, B-A and PC2, compared to those with no recorded wounds. For PWM, the opposite effect was apparent, such that IFN_{γ} response was significantly lower in animals with fresh or healed wounds, compared to those with no recorded wounds (Figure 2.5). The presence of bite wounds was not significantly related to the production of IFN_{γ} for PPD A or PC1.

Social group exposure to M. bovis

IFN_{γ} responses to PPD B, B-A, PC1 and PC2 were significantly positively correlated with the degree of exposure of the individual badger to *M. bovis*

infected badgers in their social group (Figure 2.6). No significant effects were apparent for PPD A or PWM.

Group Size

Group size was significantly negatively related to the magnitude of IFN_{γ} production for PWM and PC1. IFN_{γ} responses to PPD A were significantly positively correlated with group size (Figure 2.7).

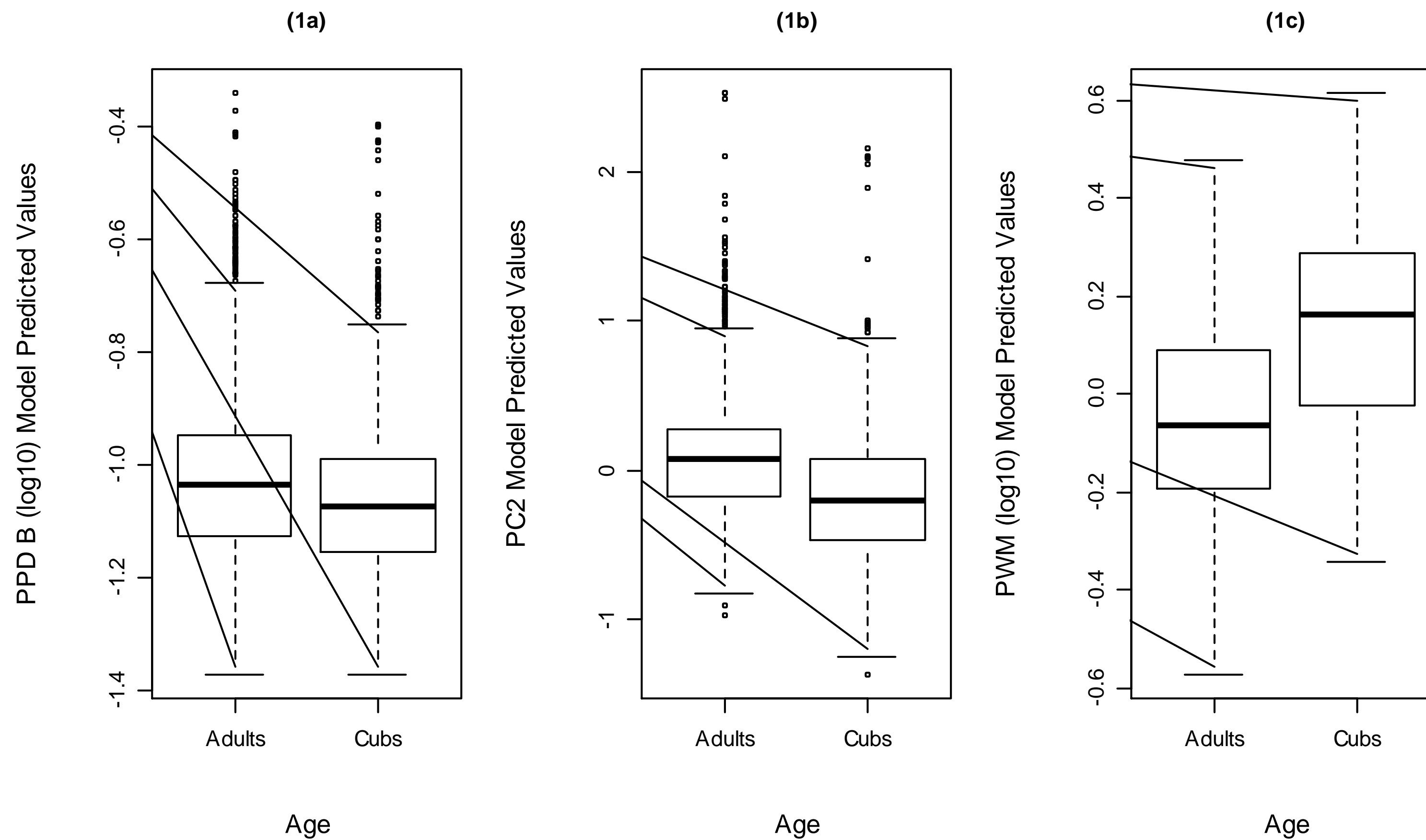


Figure 2.1. Box and whisker plots showing the significant age-related differences in IFN_{γ} production in response to (a) PPD B, (b) PC2 and (c) PWM. Values shown are the top model predicted values; the box shows the median data value (solid black line) and the lower and upper quartiles (25% and 75% of data). The top and bottom of the whiskers show the range of data excluding any outliers which are represented by the dots.

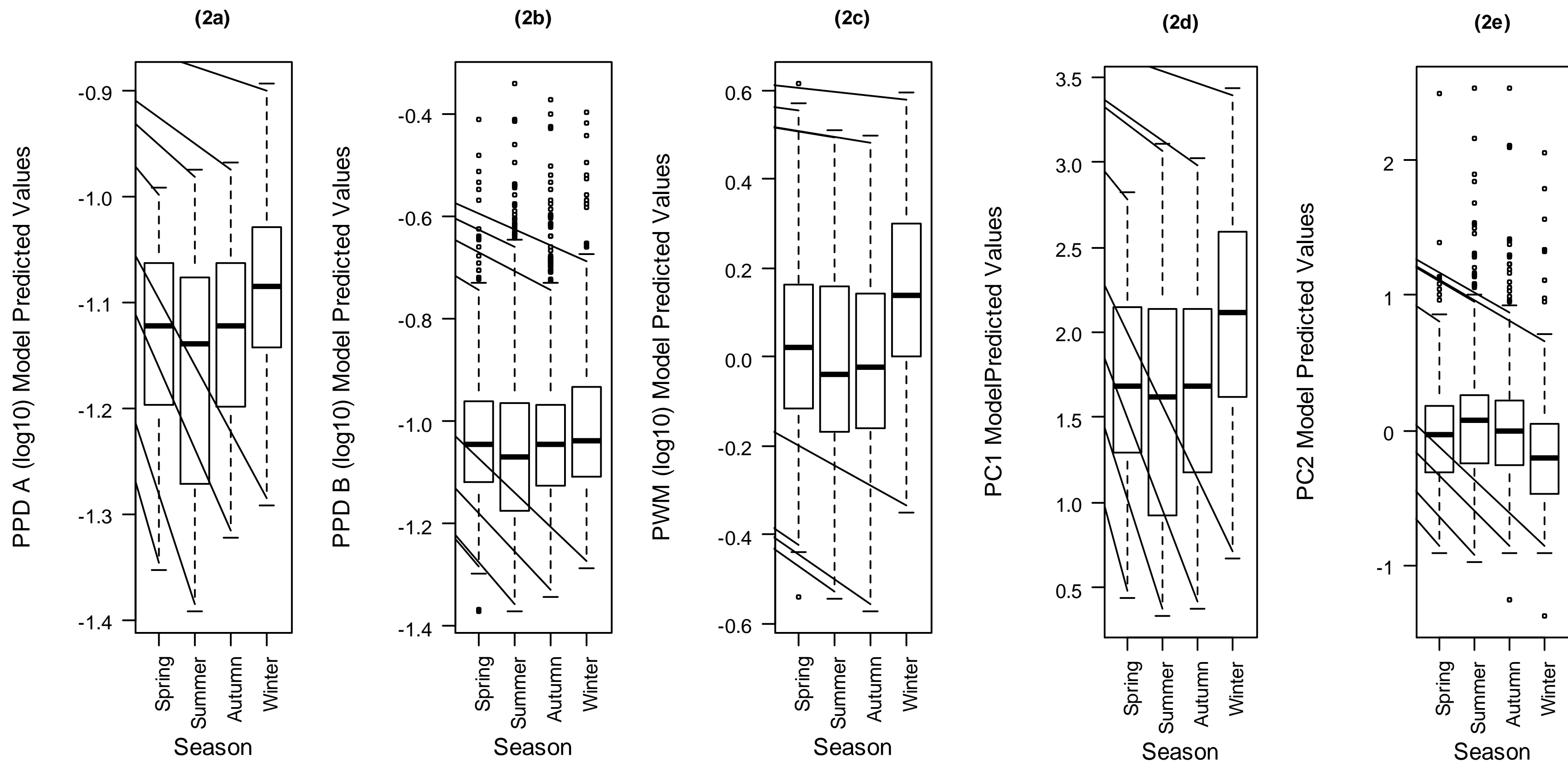


Figure 2.2 Box and Whisker plot to show the seasonal variation in IFN$_{\gamma}$ production for (a) PPD A, (b) PPD B, (c) PWM, (d) PC1 and (e) PC2. The values are the top model predicted values; the box shows the median data value (solid black line) and the lower and upper quartiles (25% and 75% of data). The top and bottom of the whiskers shows the range of data excluding any outliers which are represented by the dots.

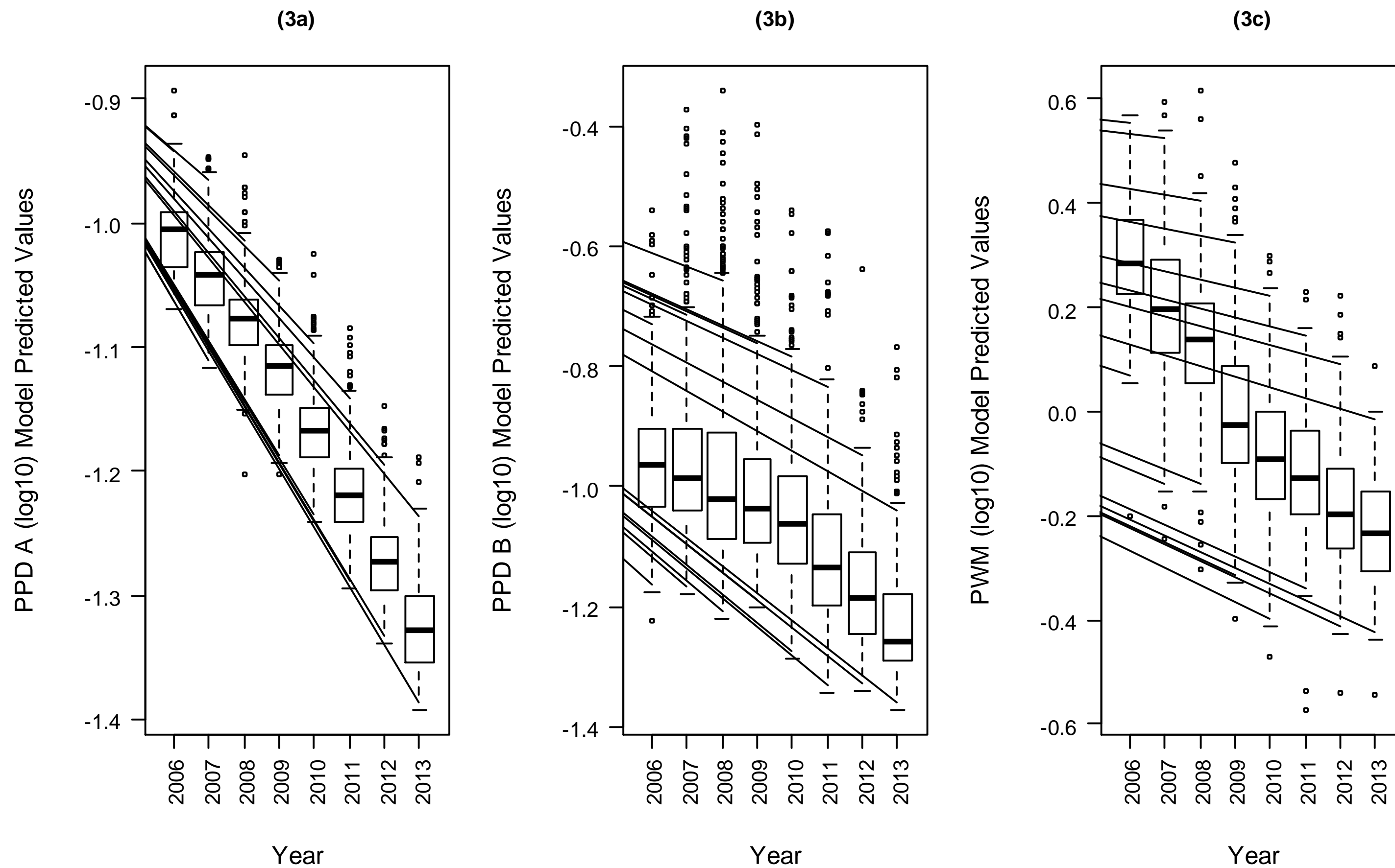


Figure 2.3. Box and Whiskers plot of the annual variation from 2006-2013 in the magnitude of IFN_{γ} responses of badgers to (a) PPD A, (b) PPD B, (c) PWM, The values are the top model predictive values; the box contains the median data values (solid black line) and the lower and upper quartiles (25% and 75% of data). The top and bottom of the whiskers shows the range of data excluding any outliers which are represented by the dots.

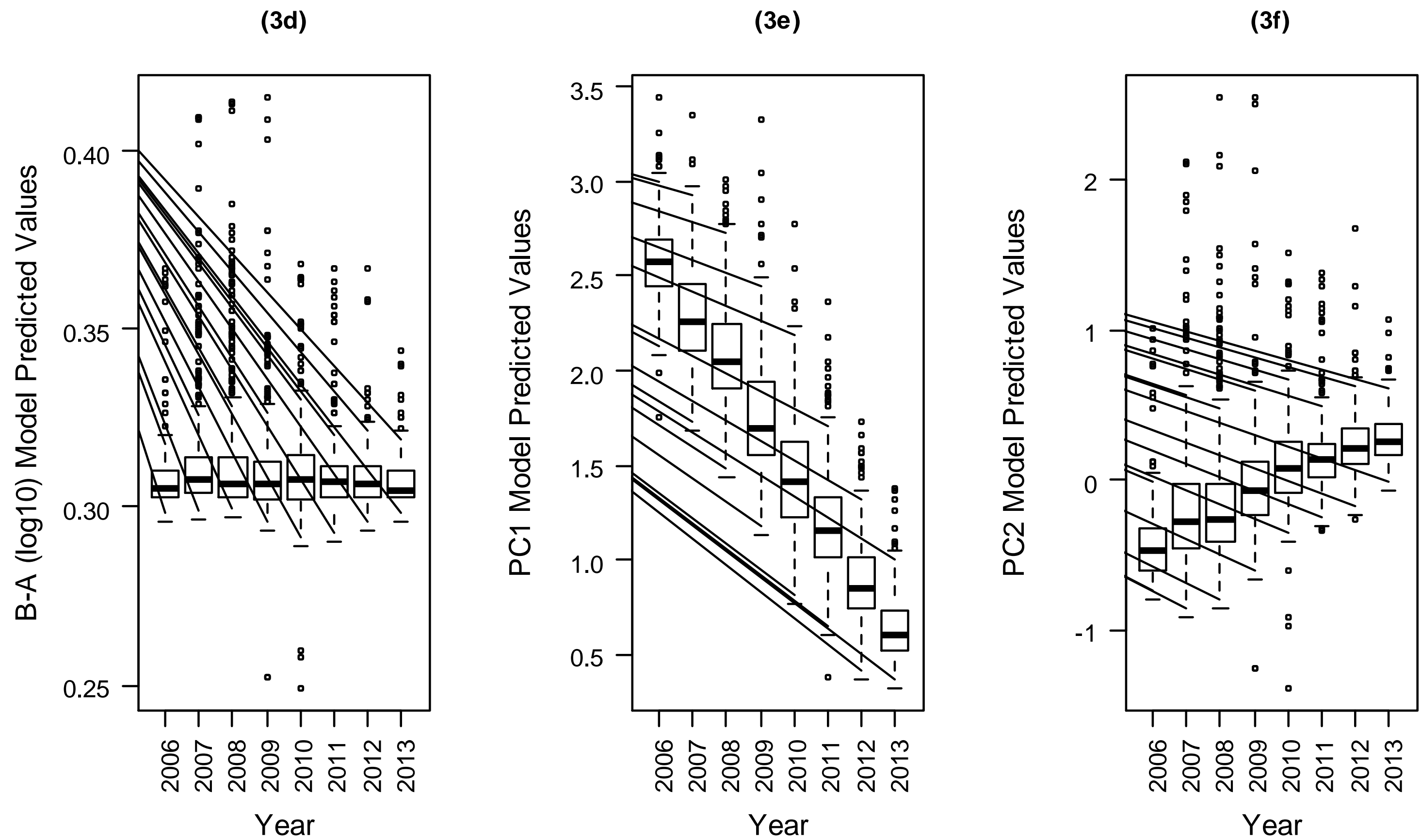
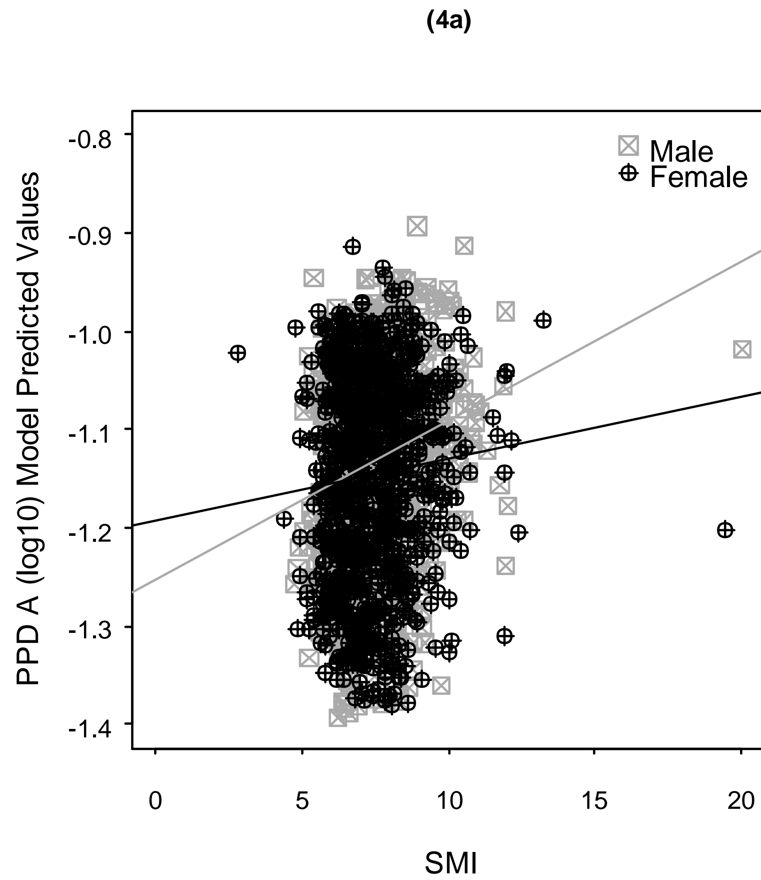


Figure 2.3. Box and Whiskers plot of the annual variation from 2006-2013 in the magnitude of IFN$_{\gamma}$ responses of badgers to (d) B-A, (e) PC1 and (f) PC2. The values are the top model predictive; the box contains the median data values (solid black line) and the lower and upper quartiles (25% and 75% of data). The top and bottom of the whiskers shows the range of data excluding any outliers which are represented by the dots.



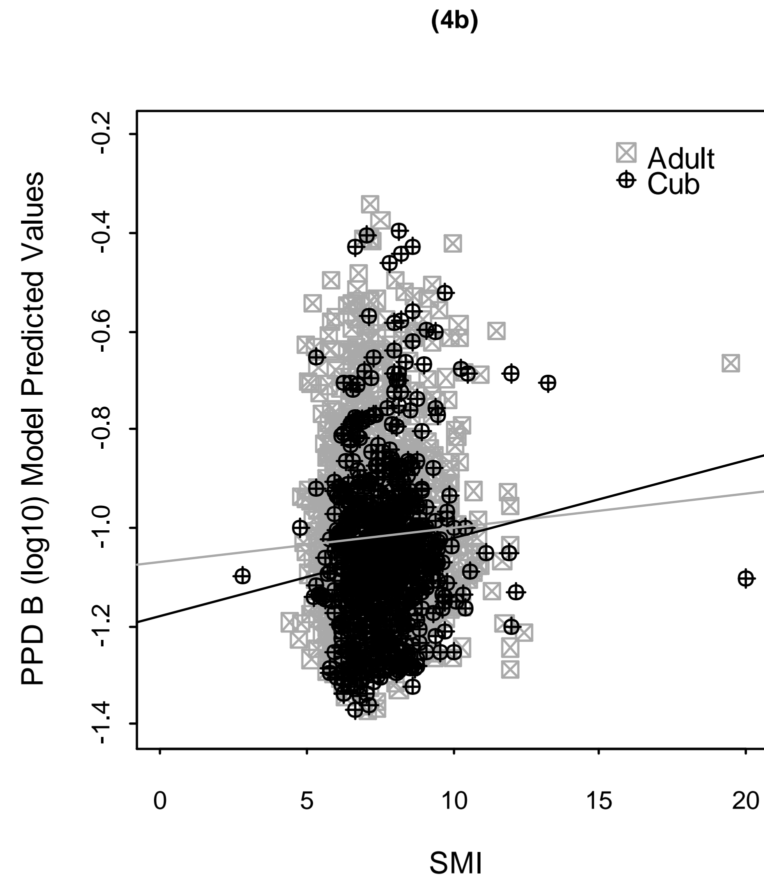
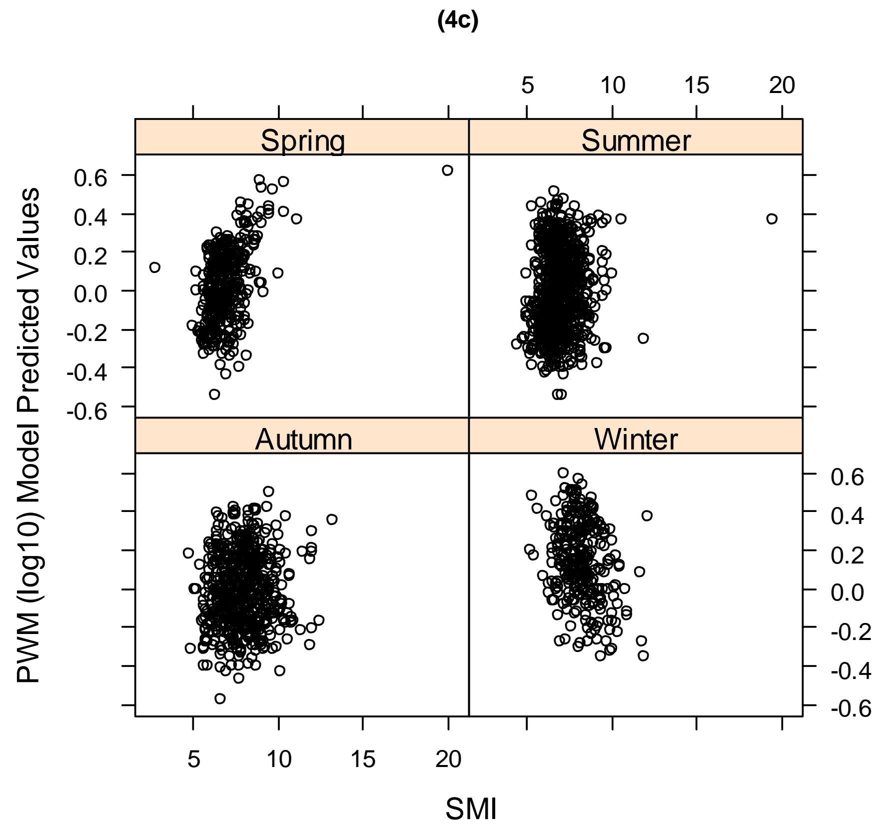


Figure 2.4. (a) Scatter plot to show the relationship between the model predicted values of IFN_{γ} response to PPD A and the body condition of male and female badgers, lines of best fit are included for each sex. (b) Scatter plot to show the relationship between the model predicted values of IFN_{γ} response to PPD B and the body condition of adult and cubs, lines of best fit are included for both age classes.



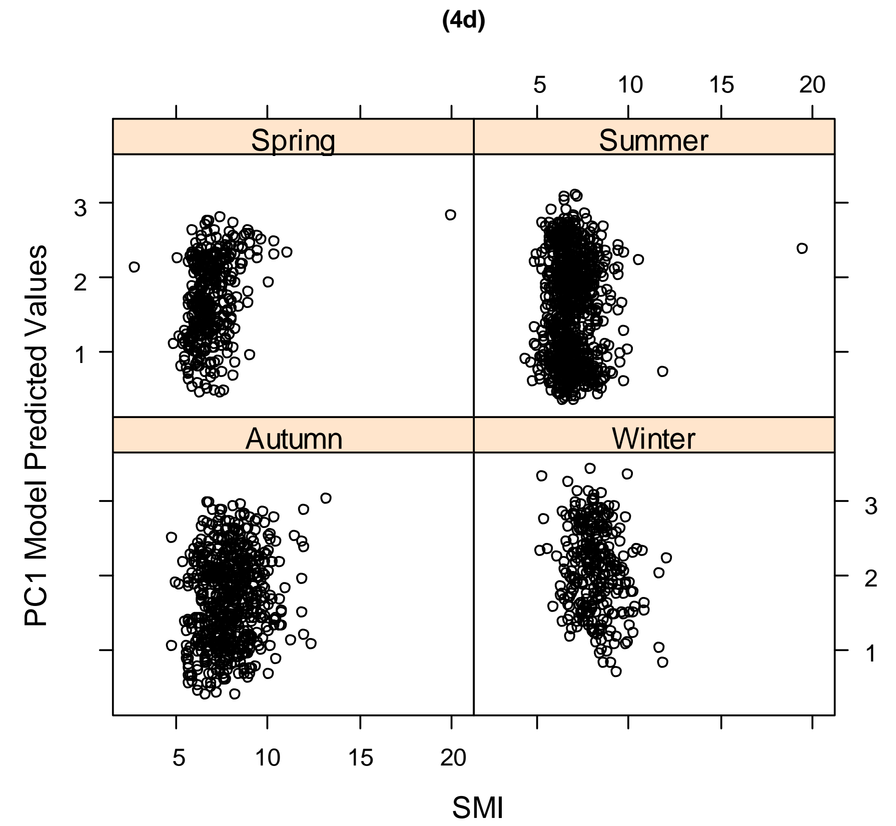


Figure 2.4. (c) Scatter plots to show seasonal variation and body condition on the model predicted values of IFN_{γ} response to PWM. (d) Scatter plots to show seasonal variation and body condition on the model predicted values of IFN_{γ} responses in the PC1 analyses.

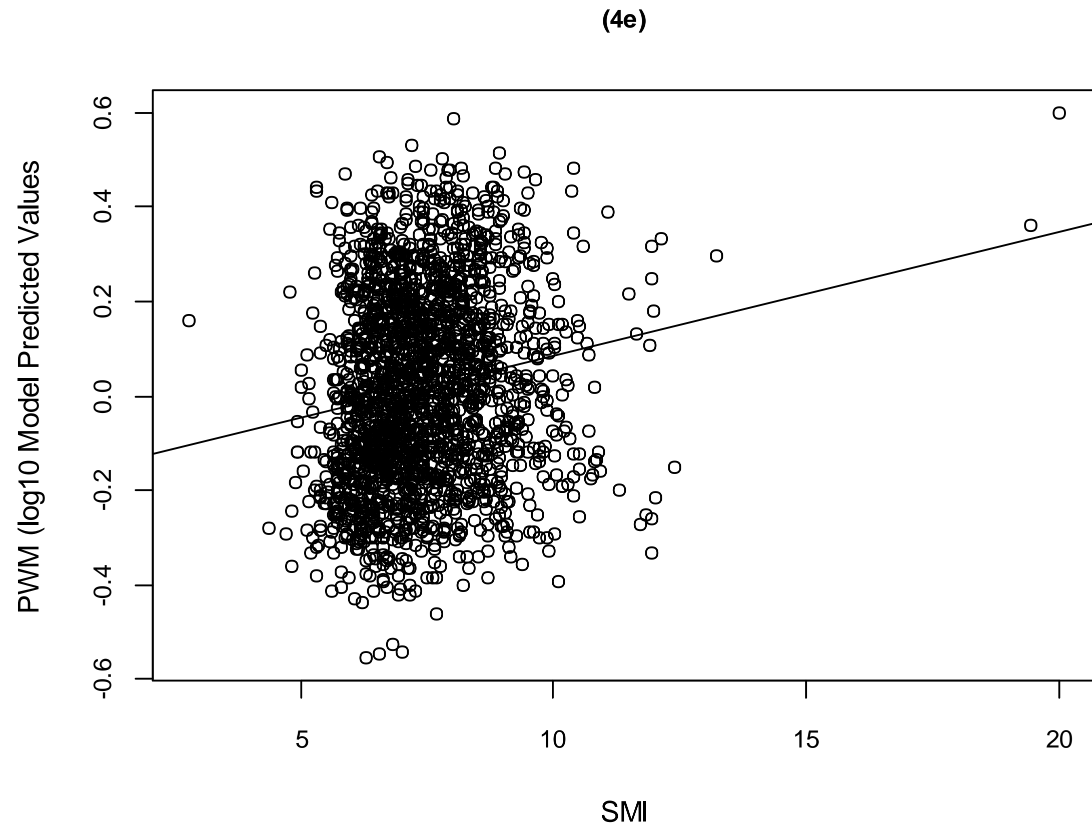


Figure 2.4. (e) Scatter plot to show the relationship between body condition and IFN_{γ} response levels to PWM, line of best fit included.

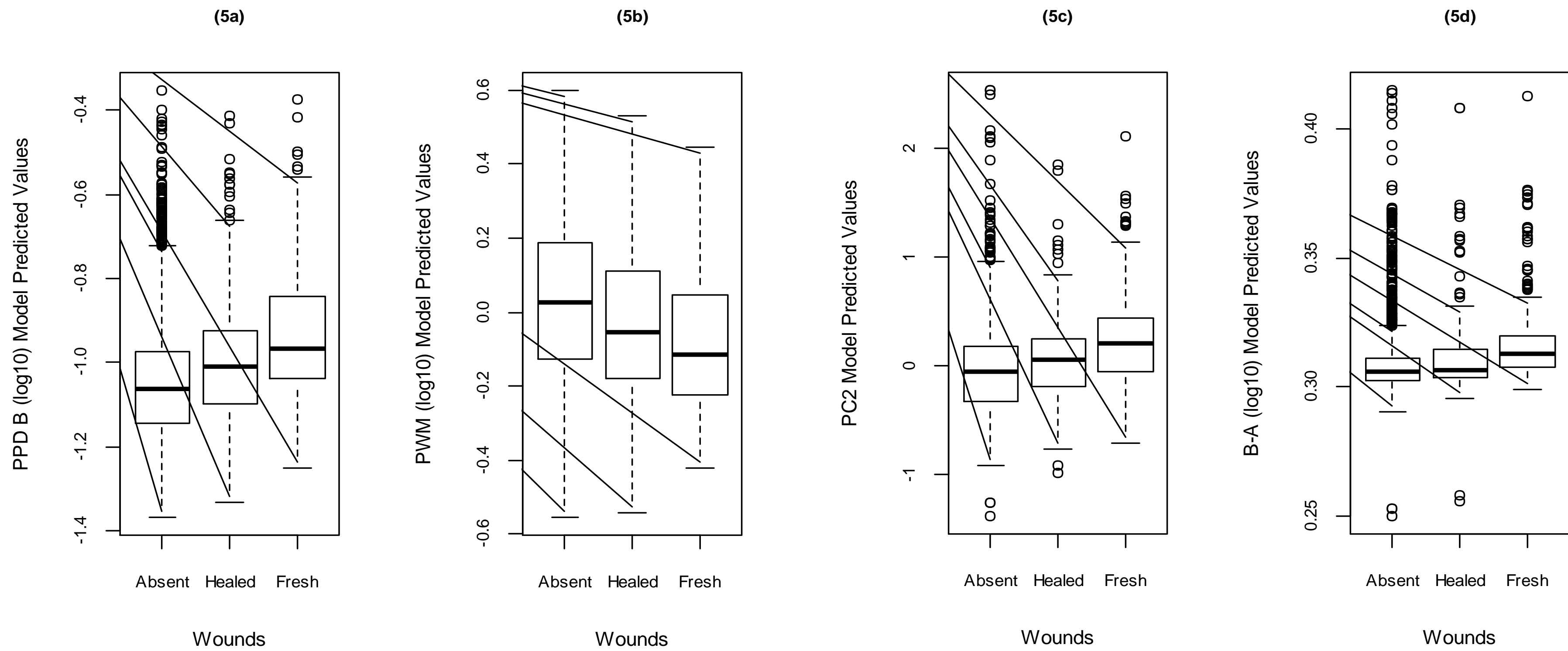


Figure 2.5. Relationships between the presence of bite wounds in badgers and IFN$_{\gamma}$ responses to (a) PPD B (b) PWM (c) PC2 and (d) B-A. The values are the top model predicted values; the box contains the median data values (solid black line) and the lower and upper quartiles (25% and 75% of data). The top and bottom of the whiskers shows the range of data excluding any outliers, which are represented by the dots.

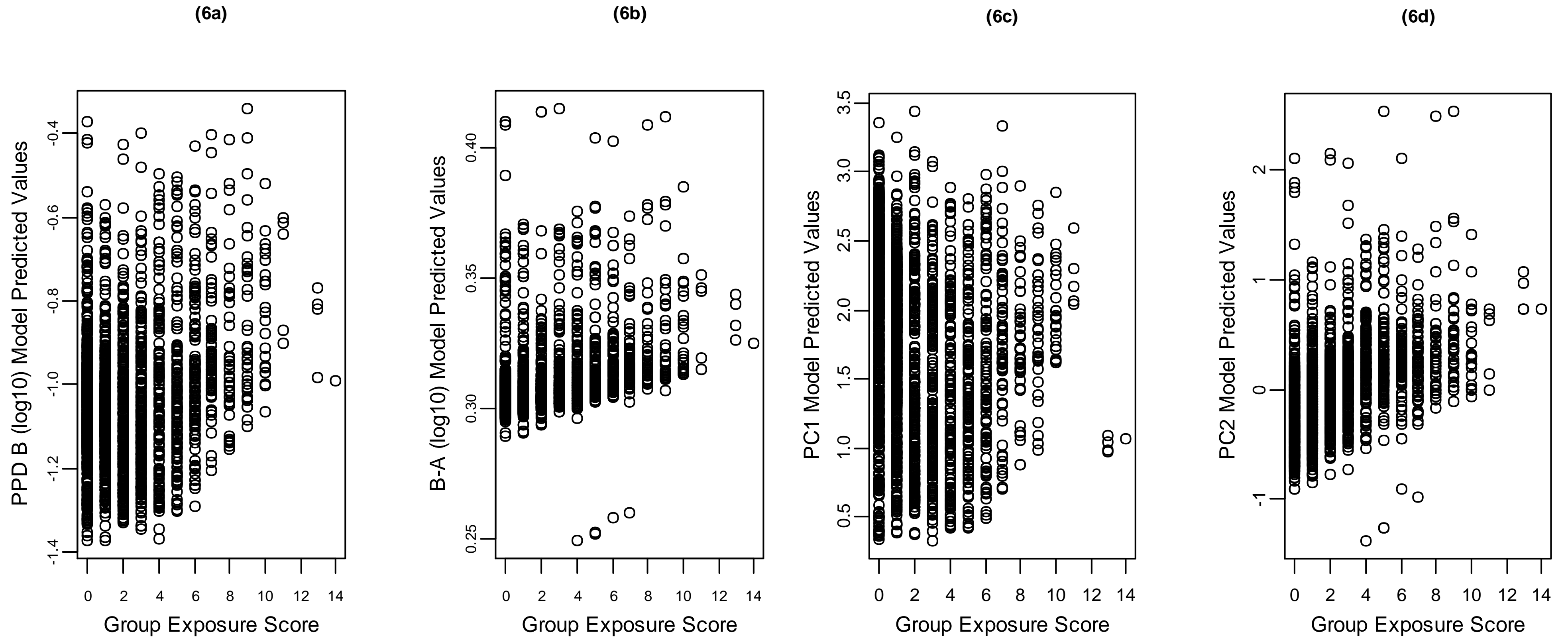
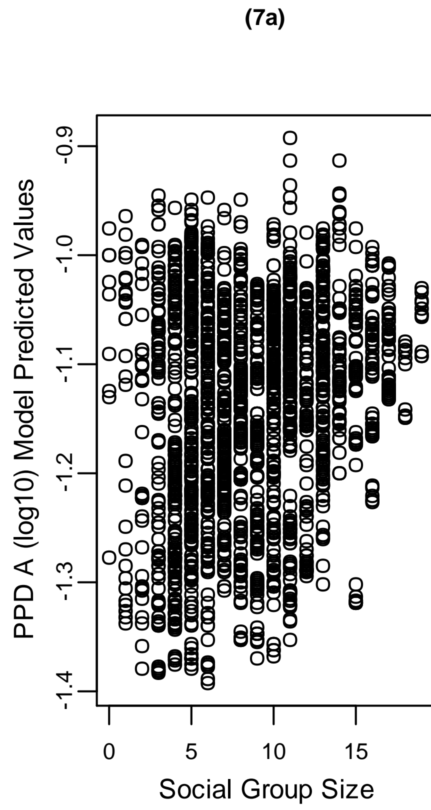
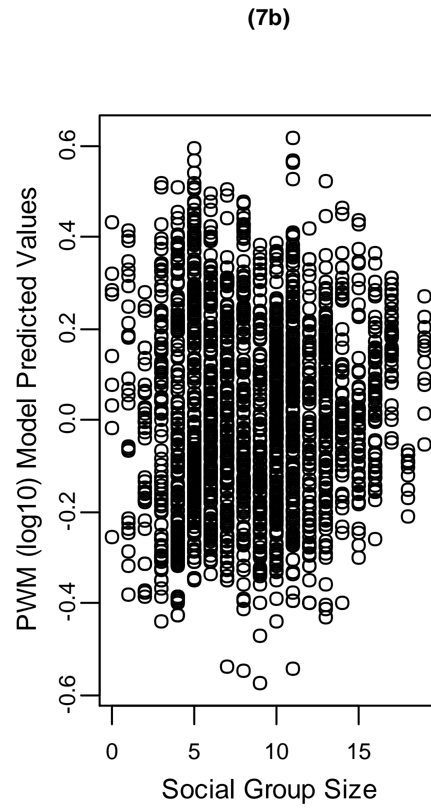


Figure 2.6. Scatter plots to show the effect of bTB exposure (based on a diagnostic test scoring system) on the model predicted values of IFN_{γ} response for (a) PPD B (b) B-A (c) PC1 and (d) PC2.





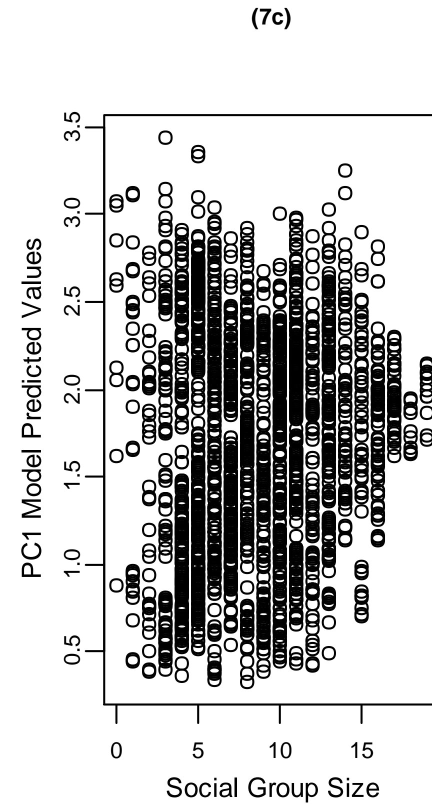


Figure 2.7. Scatter plots to show the effects of social group size on the model predicted values of IFN_{γ} response for (a) PPD A (b) PWM (c) PC1.

2.5 Discussion

By analysing the results of blood tests conducted on a large sample of individual badgers, we have revealed both intrinsic and extrinsic sources of variation in the cell-mediated immune response. Our measures of immune response are specifically assayed by IFN_{γ} production in response to lymphocyte stimulation with specific mycobacterial antigens (PPD A, PPD B) and with the more general antigen pokeweed mitogen (PWM). By using these measures of immune response and deriving parameters to indicate variation in the overall magnitude of the immune response (PC1) and the more specific components (PC2), we have highlighted the between-individual and between-state variation in immune response. This variation has a clear bearing on understanding variation in the immunological capability of individuals and, in this specific instance, on the diagnosis of important infections.

In this study, sex did not, when considered in isolation, significantly influence the magnitude of any elements of the IFN_{γ} immune response in badgers. However, the age of a badger was correlated with the cell-mediated immune response and this relationship varied depending on whether stimulation was general or specific. For example, cubs produced more IFN_{γ} than adults in response to a general stimulus (PWM). Studies in both humans and laboratory animals have identified age-related changes in the T-cell component of adaptive immunity (Castle, 2000). Gradual lifetime decreases in various cell-mediated immune responses such as lymphocyte proliferation to mitogens have been observed in humans (Murasko *et al.*, 1987; Wedelin *et al.*, 1982; Hicks *et al.*, 1983) and dogs (Greeley *et al.*, 2001). Rink *et al.* (1998) found that IFN_{γ}

released by lymphocytes is produced less in the elderly compared to young donors and is correlated with a decreased number of $CD45RA^{+}/CD8^{+}$ T-cells. Aging is associated with deterioration of the immune function and is termed as immunosenescence (Ginaldi *et al.*, 2001); this deterioration can lead to increased susceptibility to infection and disease (Castle, 2000). However, we have only distinguished two age classes in the present study and hence a more in-depth approach could be used to examine immunosenescence in badgers via a decline in PWM IFN_{γ} levels at more advanced ages.

In response to the specific stimulus of PPD B, cubs produced less IFN_{γ} than adults. The strong effect seen in PC2 further suggests that having taken account of the overall magnitude of the IFN_{γ} production (PC1), cubs are less likely to exhibit a large, specific response, in this case to PPD B. Studies of immune performance and ageing have identified a lifetime shift in the phenotype of circulating T-cells , with a decrease in naïve T-cells which are most probably involved in the general PWM response described above, and accumulation of memory T-cells ($CD45RO^{+}CD4^{+}$ cells) (Thomon and Weigle, 1989; Nijhuis and Nagelkerken, 1992), which contain normal functioning and hypofunctioning memory T-cells in older animals compared to those that remain in younger animals (Castle, 2000). This shift might help explain why adults had a greater response to the specific antigen (PPD B), as they are more likely to have been exposed to *M. bovis* and therefore developed specific memory T-cells (Chambers *et al.*, 2009). A lack of significant age-related variation in response to PPD A suggests that cubs may be no less likely than adults to have been exposed to environmental mycobacteria. The effect of age on B-A responses may be associated with the tendency for the IFN_{γ} assay for bovine

tuberculosis (which is based on B-A) to be less sensitive in cubs than in adults, which has resulted in a lower cut-off value being recommended for cubs (Chambers *et al*., 2009).

The higher prevalence of healed or fresh bite wounds found in adults may also have influenced the variation found in the specific and nonspecific immune response between adults and cubs. The presence of bite wounds affected the cell-mediated immune response differently depending on whether stimulation was general or specific. Greater IFN_{γ} responses to the specific stimuli (PPD B, B-A and PC2) in wounded animals, compared to those without bite wounds supports the notion that bite wounding may be an effective route of bTB infection for badgers and that this is associated with more rapid progression of infection than other routes of infection (Gallagher *et al*., 1998; Clifton-Hadley *et al*., 1993; Jenkins *et al*., 2008). Clifton-Hadley *et al.* (1993) showed that badgers with infected bite wounds at first diagnosis appear to have a shorter survival rate than those with *M. bovis* diagnosed from other samples indicating the likelihood that these badgers have a more rapidly disseminated form of *M. bovis*. The lower response to PWM observed in wounded animals is difficult to explain. Generalized immunosuppression after injury has been noted in studies of mice and humans with IFN_{γ} activity reported to decrease after accidental injury (Abraham and Freitas, 1989; Meldrum *et al*., 1991) and a reduction of lymphocytes and altered T-cell phenotype (Schaffer and Barbul, 1998). Lower levels of lymphocyte proliferation in response to mitogenic stimulation have been recorded in patients after surgical procedures, accidental trauma, burns and haemorrhage (Faist *et al*., 1993; O'Riordain *et al*., 1993). Stress hormones have also been found to cause adverse effects on lymphocyte function

(Schaffer and Barbul, 1998). Seasonal reproductive activity including territorial defence, bite wounding, assertion of sexual dominance, pregnancy and lactation (Neal and Cheeseman, 1996) may cause heightened physiological stress in an individual (Griffin and Thomson, 1998) perhaps contributing to the lower IFN_{γ} levels in response to PWM observed in bite wounded animals.

Significant seasonal variation was observed for both the specific and general cell-mediated responses. IFN_{γ} levels were highest during the winter months for PWM, PPD A, PPD B and the overall immune response of PC1. However PC2 (representing likely infected individuals) showed higher levels during summer. *M. bovis* can survive and may remain infectious for months in the environment (Maddock, 1993; Wilesmith *et al.*, 1986; Courtenay *et al.*, 2006). Badger setts provide the ideal conditions for *M. bovis* to survive due to their complete darkness, constant temperature and high humidity (Roper, 1992). During the winter months badgers spend more time underground in intimate association with one another, and as the duration of aerosol exposure is significantly increased in enclosed spaces, with limited air movement (Corner *et al.*, 2011), at this time of year setts may provide ideal conditions for exposure/transmission. This is consistent with the significantly higher levels of IFN_{γ} in response to PPD B found during the winter. A study in Ireland by Corner *et al* (2008) found evidence of a higher prevalence of *M. bovis* infection amongst badgers during December (winter) and significantly lower levels in May and September (spring/autumn), although no explanation was given. Such a seasonal pattern of prevalence would be consistent with the higher levels of IFN_{γ} in response to PPD B found during the winter months in the present study, but would not explain the higher levels of IFN_{γ} found in the summer related to

PC2. As PC2 represents likely infected individuals based on B-A, a possible explanation may be the higher IFN_{γ} levels found in response to PPD A. False negative results to the IFN_{γ} test have occurred in studies of cattle due to high levels of IFN_{γ} in response to PPD A as well as PPD B (Rhodes *et al.*, 2000; Schiller *et al.*, 2010). Other studies in cattle and badgers have also revealed that IFN_{γ} responses to PPD A may exceed those to PPD B at an early time point after *M. bovis* infection and therefore the time when the diagnostic test is performed may play an important role in test accuracy (Rhodes *et al.*, 2000; Lesellier *et al.*, 2008; Schiller *et al.*, 2010). These results show a weakness in the use of tuberculins for the diagnosis of *M. bovis* due to the potential cross-reactivity of PPD A and PPD B and risk of co-infection with *M. bovis* and other mycobacteria *(*Aagaard *et al.*, 2010). The PC2 results could also reflect seasonal variation in badger physiology, particularly that related to reproductive behaviour. Female badgers undergo pregnancy during the winter months and give birth in late winter/early spring. Mating can start soon after the birth of cubs and before weaning which takes place 12 weeks after birth (Roper, 2010; Neal and Cheeseman, 1996). Stress caused by reproductive activity (previously described), coupled with poor body condition and nutritional stress after the winter period of inactivity could have a negative impact on the immune system. Studies of food restriction and body mass changes in birds have shown that food restriction caused a decrease in the cell-mediated immune response to mitogens that were significantly correlated with the percentage of body mass lost by each bird (Alonso-Alvarez and Tella, 2001). A reduction in immunocompetence may lead to reactivation of latent *M. bovis* infection into progressive disease (Gallagher and Clifton-Hadley, 2000; Gallagher *et al*, 1998)

or heightened susceptibility to establishment of disease following exposure, which would be consistent with higher levels of IFN_{γ} production in the summer.

Badgers have the highest body condition scores coming into winter after accumulating weight ready for the period of inactivity. Enhanced body condition is achieved through increased food uptake during the previous months when food is more abundant and this may contribute to a healthier immune system. Small mammals and birds have shown elevated immune activity during winter (Nelson and Demas, 1996; Nelson, 2004), which may be indicative of the presence of greater exposure to infection or an enhancement of disease avoidance (Norris and Evans, 2000) during this challenging period. The present study revealed a significant positive correlation between body condition and IFN_{γ} responses to PWM and PC1, thus implying that animals with a higher IFN_{γ} response to PWM may have a “more active” immune system. However, these analyses also show that during the winter months PWM and PC1 levels are higher in animals in poorer body condition. Immune responses require fuel in the form of nutrients and energy, which can lead to loss of body weight if the response is prolonged (Lochmiller and Deerenberg, 2000). The raised PWM IFN_{γ} levels found during the winter months coupled with the shortage of food and reproductive state may cause a loss in body condition in some individuals and perhaps explain why a negative correlation was observed. However, this is speculative and suggests that further investigation may be required. The body condition of badgers does not appear to have a significant effect on the antigen specific stimuli (PPD A and PPD B) or within the PC1 and PC2 responses. However the interaction between age and body condition of badgers and the PPD B response revealed that cubs produced significantly higher levels

compared to adults with an increase in body condition. In addition, males produced significantly more IFN_{γ} to PPD A than females with increasing body condition. This is hard to explain but identifies the complex interactions between investment and activity in immunity and infection between the sexes.

The year on year decline in IFN_{γ} levels shown in all analyses apart from PC2 (annual increase possibly due to the annual decline in PWM and PPD A accentuating the higher PPD B responses) is also difficult to explain but could be attributed to test performance, a physiological or environmental reason or some combination of these factors. Investigation into any changes in test procedure or reagent batches would be required to see if test performance may have changed over time.

Increased stress may be experienced by individuals living in larger social groups if there is a higher level of intra-specific competition for food, territorial defence and reproduction. Immune suppression has been associated with stress hormones in several species of vertebrate (Nelson *et al.*, 2002), and may explain the negative correlation between group size and IFN_{γ} levels in response to PWM and within the PC1 analyses observed in this study. A study by Beirne *et al.* (2014) has also shown evidence of the trade-off between intra-sexual competition and somatic cell maintenance in badgers so this may also be a contributing factor. There was no significant association apparent between group size and the specific *M. bovis* response variables (PPD B, B-A and PC2) however in the present study badgers living in social groups with a high level of exposure to *M.bovis* showed raised levels of IFN_{γ} to the antigen specific stimulus. This is to be expected as close associations between badgers of the

same social group including mutual grooming and the use of the same main setts may enhance the spread of infection.

The present study has provided an insight into the effect of various intrinsic factors on the cell-mediated immune responses of badgers. Results have revealed a wide range of explanatory variables with the potential to significantly influence the PWM IFN_{γ} response which determines the state of the 'naïve' T-cell population. Those variables that have been shown to significantly influence the PPD B IFN_{γ} response provide evidence of what intrinsic factors may contribute to the likelihood of *M. bovis* infection/exposure.

A number of host and environmental factors may influence an individual's immune response and the performance of the diagnostic test, including the intrinsic factors observed in this study. Clearly, incorporation of knowledge of these intrinsic and extrinsic factors into diagnostic determination of the bTB infection status has potential to improve test performance, by controlling for variation in test outcomes that does not arise from infection status. There are however other factors which may influence the immune response and diagnostic test outcomes that were not included in the present study. Such factors include the influence of other infections, disease severity, stress, resource availability and their interactions. Further investigation into intrinsic factors that specifically influence the *M. bovis* specific IFN_{γ} response would be required if they were to be used as a tool to assist in determining the disease status of badger individuals/groups in TB eradication programmes.

2.6 Acknowledgements

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2.8 Appendix I

Table 2.3. Summary table of top models to explain the influence of various intrinsic factors on each response variable. Inclusion of a given variable is indicated by the symbol +, the ΔAICc and Delta value are given to represent the fit of models and the weight to show the probability of the given model best explaining the data.

Response Variable	Sex	Age	Season	Wounds	Year	SMI	GS	bTB exp	Age *Sex	Age* Season	Age* Wounds	Age* SMI	Age * GS	Sex * Season	Sex* Wounds	Sex* SMI	Sex* GS	Season* SMI	*df*	ΔAICc	Delta	Weight
PPD A	+	+	+		+	+	+		+	+						+		+	20	-1626.69	0.00	0.27
	+	+	+		+	+	+		+	+						+			17	-1625.38	1.30	0.14
	+	+	+		+	+	+	+	+	+						+		+	21	-1625.34	1.35	0.14
	+	+	+		+		+		+	+									15	-1625.05	1.64	0.12
	+	+	+		+	+	+		+	+			+			+		+	21	-1624.93	1.76	0.11
	+	+	+		+	+		+	+	+						+		+	20	-1624.77	1.92	0.11
	+	+	+		+		+			+									14	-1624.72	1.97	0.10
PPD B		+	+	+	+	+	+	+				+						+	18	581.54	0.00	0.17
		+	+	+	+	+	+	+				+							15	581.81	0.27	0.15
		+	+	+	+	+		+				+						+	17	582.23	0.69	0.12
		+	+	+	+	+		+				+							14	582.53	0.99	0.11
	+	+	+	+	+	+	+	+				+					+	+	20	582.92	1.38	0.09
	+	+	+	+	+	+	+	+				+						+	19	583.13	1.59	0.08
	+	+	+	+	+	+	+	+				+				+	+	+	21	583.19	1.65	0.08
	+	+	+	+	+	+	+	+				+							16	583.30	1.76	0.07
	+	+	+	+	+	+	+	+				+				+		+	20	583.42	1.88	0.07
	+	+	+	+	+	+	+	+				+					+		17	583.49	1.95	0.07

PWM	+	+	+	+	+	+	+									+		+	18	1250.84	0.00	0.11
	+	+	+	+	+	+	+								+	+		+	20	1251.01	0.25	0.09
	+	+	+	+	+	+	+	+								+		+	19	1251.36	0.52	0.08
	+	+	+	+	+	+	+	+							+	+		+	21	1251.72	0.88	0.07
	+	+	+	+	+	+	+					+				+		+	19	1252.10	1.26	0.06
	+	+	+	+	+	+	+									+	+	+	19	1252.19	1.35	0.05
	+	+	+	+	+	+	+											+	17	1252.23	1.39	0.05
	+	+	+	+	+	+	+					+			+	+		+	21	1252.37	1.53	0.05
	+	+	+	+	+	+	+								+	+	+	+	21	1252.38	1.54	0.05
	+	+	+	+	+	+	+							+		+		+	21	1252.45	1.61	0.05
	+	+	+	+	+	+	+	+				+				+		+	20	1252.48	1.64	0.05
	+	+	+	+	+	+	+	+								+	+	+	20	1252.70	1.86	0.04
	+	+	+	+	+	+	+		+						+	+		+	21	1252.72	1.88	0.04
	+	+	+	+	+	+	+				+					+		+	20	1252.73	1.89	0.04
	+	+	+	+	+	+	+	+										+	18	1252.74	1.90	0.04
	+	+	+	+	+	+	+		+							+		+	19	1252.77	1.93	0.04
	+	+	+	+	+	+	+							+	+	+		+	23	1252.79	1.95	0.04
	+	+	+	+	+	+	+	+						+		+		+	22	1252.81	1.97	0.04
PC1		+	+		+	+	+	+		+								+	18	6113.46	0.00	0.26
		+	+		+		+	+		+									14	6113.79	0.33	0.22
	+	+	+		+	+	+	+		+								+	19	6114.69	1.24	0.14
		+	+		+	+	+	+		+		+						+	19	6114.84	1.39	0.13
	+	+	+		+	+	+	+		+							+	+	20	6115.04	1.59	0.12
	+	+	+		+		+	+		+									15	6115.61	1.61	0.12
PC2	+	+	+	+	+			+	+										14	5247.87	0.00	0.34
	+	+	+	+	+			+	+	+									17	5248.88	1.01	0.21
	+	+	+	+	+			+											13	5249.17	1.30	0.18
		+	+	+	+			+											12	5249.55	1.68	0.15
	+	+	+	+	+	+		+	+										15	5249.76	1.89	0.13
B-A	+			+	+			+											9	-8378.21	0.00	0.14
	+	+		+	+			+	+										11	-8377.77	0.43	0.11
	+	+		+	+			+											10	-8377.49	0.72	0.10

+		+	+		+	+			10	-8377.47	0.73	0.10
+		+	+			+		+	11	-8377.11	1.10	0.08
+	+	+	+		+	+	+		12	-8376.90	1.31	0.07
+	+	+			+	+	+		11	-8376.61	1.60	0.06
+		+	+		+	+		+	12	-8376.49	1.72	0.06
+	+	+	+			+	+	+	13	-8376.44	1.77	0.06
+	+	+	+			+		+	12	-8376.41	1.80	0.06
		+	+			+			8	-8376.35	1.85	0.06
+		+	+	+		+			10	-8376.33	1.88	0.05
+	+		+			+			8	-8376.20	1.88	0.05

2.9 Appendix II

Table 2.4. Statistical output for all significant results. The top levels for each factor variable in the statistical analyses are: sex = female, age = adults, season = spring, wounds = absent. All other variables are continuous and therefore no separate categories.

Fixed Effects	2.5%CI	97.5%CI	Relative Importance	Significance
PPD A				
(Intercept)	-1.159	-1.114		
Season Winter	0.029	0.103	1.00	+++
Group Size (GS)	0.000	0.032	0.89	+
Year	-0.205	-0.174	1.00	+++
Sex:SMI	0.005	0.064	0.78	+
PPD B				
(Intercept)	-1.09	-1.03		
Age	-0.09	-0.03	1.00	+++
Season Winter	0.05	0.16	1.00	+++
Wounds Fresh	0.02	0.11	1.00	++
Group Exposure (GE)	0.09	0.16	1.00	+++
Year	-0.23	-0.17	1.00	+++
Age:SMI	0.00	0.11	1.00	+
PWM				
(Intercept)	0.04	0.13		
Age	0.08	0.15	1.00	+++
Season Winter	0.03	0.16	1.00	++
Season Autumn	-0.17	-0.08	1.00	+++
Season Summer	-0.11	-0.03	1.00	++
Wounds Fresh	-0.13	-0.02	1.00	+
Group Size (GS)	-0.09	-0.01	1.00	++
SMI	0.01	0.16	1.00	+
Year	-0.35	-0.29	1.00	+++
Season Winter:SMI	-0.31	-0.08	1.00	+++
Season Autumn:SMI	-0.18	-0.00	1.00	+
B-A				
(Intercept)	0.31	0.31		
Wounds Fresh	0.00	0.01	0.95	+
Group Exposure (GE)	0.01	0.01	1.00	+++
Year	-0.01	-0.00	1.00	+
PCA Component 1				
(Intercept)	1.61	1.90		
Season Autumn	-0.40	-0.08	1.00	++
Season Winter	0.22	0.71	1.00	+++
Group Exposure (GE)	0.12	0.35	1.00	+++
Group Size (GS)	-0.26	-0.04	1.00	++
Year	-1.33	-1.12	1.00	+++
Season Winter:SMI	-0.86	-0.10	0.66	+
PCA Component 2				
(Intercept)	9.42	9.64		
Age	- 0.30	-0.01	1.00	+
Season Autumn	0.03	0.26	1.00	+
Season Summer	0.00	0.21	1.00	+
Wounds Fresh	0.06	0.33	1.00	++
Group Exposure (GE)	0.47	0.33	1.00	+++
Year	0.24	0.42	1.00	+++

CHAPTER 3 Development of a technique for blood collection from unanaesthetised badgers (*Meles meles*) and application of the Dual-Path Platform VetTB diagnostic test in the field.

3.1 Abstract

Existing methods of *Mycobacterium bovis* diagnosis in live badgers require the collection of blood or clinical samples from captured and anaesthetised animals. The development of a simple technique to collect and test blood from unanaesthetised badgers in the field would be a valuable tool for disease surveillance and management. This study demonstrated proof of concept of a method of capillary blood collection which can be used in the field to test for the presence of *Mycobacterium bovis* infection in badgers. Blood collection was achieved by using a bespoke restraint cage allowing safe access to the badger's hind leg. A small incision was made in the main pad with a lancet device and capillary ooze collected. The blood collection method was developed on 57 badgers of which 41 had a valid Dual-Path Platform VetTB result in the field and laboratory. We have shown that the accuracy of the DPP VetTB test carried out in the controlled conditions of the laboratory cannot be replicated when testing under field conditions. Only a moderate agreement was observed between tests carried out in the field and the laboratory suggesting that stochastic conditions in the field may affect TB diagnostic outcomes. This is further supported by receiver operating characteristic curves revealing

significantly more accurate results in the laboratory than in the field, when using a more sensitive interferon gamma assay as reference.

3.2 Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* is a serious zoonotic disease that has a significant impact on livestock health and production (Defra, 2011), which in turn results in substantial costs to the UK tax payers and the cattle industry. The European badger (*Meles meles*) was first recognised as a wildlife host for *M. bovis* in 1971 during investigation of a bTB outbreak on a farm in Gloucestershire (Muirhead *et al*., 1974). Further research identified badgers as a maintenance host and a significant source of *M. bovis* infection in cattle (Krebs *et al.,* 1997).

From 1973 until 1997, and again since 2011(Defra, 2011) badger culling has formed part of bTB control policy in England. In cattle, bTB incidents have increased in both number and geographical spread since the 1980s (Krebs *et al*., 1997). Results from the Randomised Badger Culling Trial (RBCT) showed that widespread proactive culling reduced cattle TB incidence inside the cull zone but increased the incidence in the neighbouring unculled areas. This was believed to be caused by increased movement in badger populations disrupted by culling which extended into adjacent areas resulting in increased transmission between badgers and from badgers to cattle (Woodroffe *et al*., 2006). Badger culling has not discriminated between infected and uninfected animals, which may have resulted in a large number of uninfected animals

being killed (Buddle *et al*, 2000) and is therefore a less attractive option from an animal welfare standpoint. In addition, removal of fewer animals might be expected to reduce the level of disruption caused by culling and hence the negative epidemiological effects (Bielby *et al.*, 2014).

In 1994 a trial was carried out using a diagnostic test on live badgers to explore this approach for targeted culling of infected badgers (Woodroffe *et al.*, 1999). Unfortunately identifying and removing infected social groups was severely constrained by the low sensitivity of the test and the trial was abandoned.

Vaccination of wildlife against *M. bovis* infection is another approach that aims to reduce the spread of infection amongst badgers and to other animals (Buddle *et al.*, 2000; Chambers *et al.*, 2011; Carter *et al.*, 2012). With the development of a suitably sensitive diagnostic test that could be used at trap-side, a test vaccinate/remove (TVR) programme might be a more attractive option, allowing test positive animals to be removed and test negative animals to be vaccinated.

Diagnostic tests such as bacterial culture of clinical samples and the interferon gamma test (IFN_{γ}) are available for the detection of *M. bovis* in badgers but would not be suitable for field application. Both tests require a specialist laboratory environment and skilled personnel to carry out the procedure, with a minimum test turn-around time of several weeks for culture (Gallagher and Horwill, 1977) and 48hrs for the IFN_{γ} (Dalley *et al.*, 2008). The Dual-Path Platform VetTB point-of-care test is an immunochromatographic test for rapid serological detection of bTB using innovative dual-path platform (DPP) technology (Chembio, Medford, NY, USA). The VetTB test has two

nitrocellulose strips that are connected in a T-shape inside the cassette device, this allows independent delivery of sample and antibody detecting reagent (protein A/G hybrid conjugated to colloidal gold particles) (Boadella *et al.*, 2011). Another advantage of the VetTB test is that it detects two target diagnostic antigens of *M. bovis* (MPB83, ESAT6/CFP10) on two separate lines. Line one contains antigen MPB83 and line two ESAT6/CFP10 (Chambers, 2013). In the presence of antibodies specific to these antigens, a line is produced the intensity of which can be measured in reflectance light units (RLU). The RLU values correspond to the amount of antibodies present in the sample and a cut-off value can be applied to determine a positive/negative result. In addition, results can be read by eye.

This test could be used at trap-side because a result can be achieved within 20 minutes, and its application has been described in other wildlife species (Greenwald *et al.*, 2009, Buddle *et al.*, 2010 and Boadella *et al.*, 2011). This would enable captured badgers to be kept in cage traps for a relatively short period of time, during which a serological test could be carried out. However, in order to conduct the test in the field, it is necessary that a robust protocol be developed for the collection of a viable blood sample from the unanaesthetised badger.

Another consideration when developing this protocol concerns test performance in the field. The manufacturer's instructions state that the test needs to be carried out on a flat surface at temperatures of 18 to 30^{0}C with good lighting in order to interpret the results more accurately. Considering the test has been developed to perform optimally under these controlled conditions, replicating

them in the field may affect test accuracy due to environmental factors such as temperature, uneven terrain and low light levels. In addition, contamination of the sample may occur due to the nature of blood collection in the field. Blood taken from an unanaesthetised badger will not be as aseptic to that taken from an animal under anaesthetic in a controlled environment.

The present study describes the development of a method for capillary blood collection on 57 live and unanaesthetised badgers and its application on the VetTB test in the field. The study then compares VetTB test results from 41 capillary blood samples successfully taken and tested in the field from unanaesthetised badgers with those from venous whole blood samples taken from the same animals on the same occasion but under controlled conditions, i.e. under anaesthetic and the tests run in a laboratory.

3.3 Method

Overview

Badgers were trapped as part of the long-term project at Woodchester Park, Gloucestershire, UK (see Delahay *et al.*, 2000; Vicente *et al.* 2007). At each of ten scheduled trapping events from May 2014 to January 2015, trap-side collection of blood was attempted from a selection of trapped but unanaesthetised badgers. If blood collection was successful then the sample was tested for the presence of antibodies against *M. bovis* antigens MPB83, ESAT6 and CFP10 using the VetTB test (Chembio Diagnostic Systems Inc. USA).

The use of a novel badger restraint cage (Figure 3.1 & 3.2) developed by the Animal and Plant Health Agency (APHA) (previously Food and Environment Research Agency) in partnership with Envisage PMS Ltd, allowed the operator to gain access to the hind leg of an unanaesthetised badger without risk of injury to either operator or restrained badger. Each trapped badger was transferred from the trap into the restraint cage. A hind leg was then gently taken out of the cage through a window in the cage and the main pad of the foot was cleaned with water soaked cotton wool and dried using a paper towel. A small incision was then made by applying the lancet of choice to the skin of the main pad (Figure 3.3). If capillary ooze was produced from this incision, 10 µl (or as close to this volume as possible) was collected in a heparinised capillary tube (Figure 3.4) and placed directly onto the VetTB test well (Figure 3.5). The badger was then taken back to a dedicated sampling facility as part of the routine trapping project to undergo further sampling under anaesthesia (Delahay *et al.*, 2013). At the sampling facility, whole blood was collected in a 4 ml BD heparin vacutainer tube and tested using the identical VetTB test in the adjoining laboratory.

Figure 3.1. Novel badger restraint cage.

Figure 3.2. Novel badger restraint cage in use.

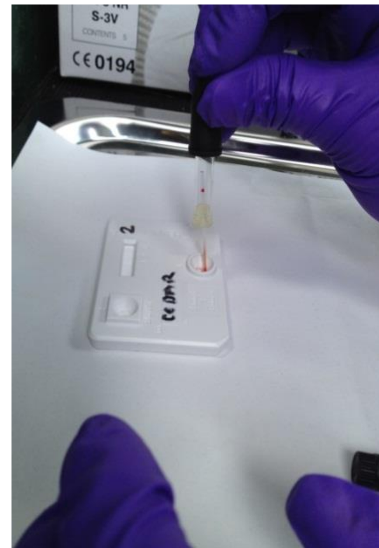

Figure 3.3. Hind leg access and lancet use.

Figure 3.4. Capillary ooze collection from incision site.

Figure 3.5. Blood application to VetTB test sample well.

Lancet for Skin Puncture

To create sufficient capillary ooze from which to take a sample, an effective method of making an incision in the central depression of the main pad of the badger’s hind foot (site determined by trials carried out by APHA on both wild

and captive badgers) was required. Four different lancets were considered. These included a MiniCollect® safety lancet, penetration depth 2 mm with blade with and without safety casing (Greiner Bio-One, product code 450429, Figure 3.6a and 3.6b), and a 2 mm needle lancet (Sterimatic Worldwide Ltd, Figure 3.6c). Both of these lancets are used for blood sampling in humans. The two other lancets sourced are used to draw blood from the saphenous vessel of mice and rats; these consisted of a 4 mm and 6 mm goldenrod animal lancet with a stainless steel triangular blade (World Precision Instruments, Inc., product code 504540 and 504553. Figure 3.6d & e). For the human lancets it was decided that the 2 mm blade in the safety casing would not create enough depth to penetrate the skin of the badger's pad so the blade was removed from the safety casing.

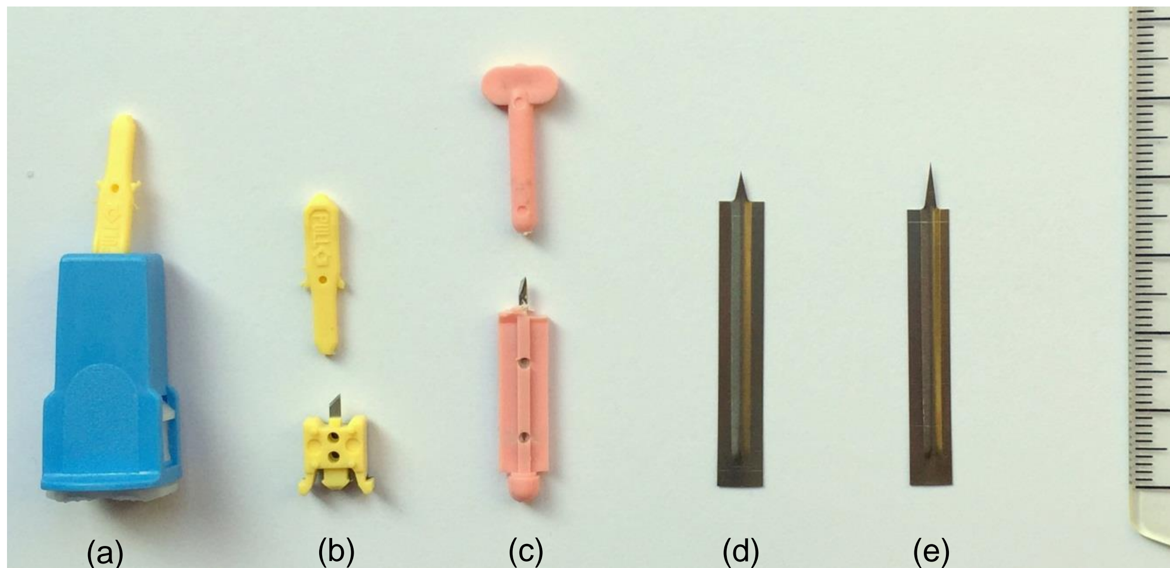

Figure 3.6. Various lancet devices trialled; (a) 2 mm blade with safety casing, (b) 2 mm blade with safety casing removed, (c) 2 mm needle lancet, (d) 4 mm goldenrod animal lancet, and (e) 6 mm goldenrod animal lancet.

Blood Collection Apparatus

A sample of 10 μl of whole blood was required to carry out the VetTB test as per manufacturer's instructions (Chembio Diagnostic Systems Inc, 2012). Various collection vessels were sourced including plastic pastettes, capillary tubes and a vacuum device. Four types of plastic pastette were trialled with drop volume ranging from 20 μl to 25 μl. Due to the larger drop volume of these pastettes any blood collected is dispensed into a heparin microtainer blood collection tube (BD, product code 365965). Pastettes included a paddle pastette (131 mm length, 25 μl/drop; Alpha Laboratories, product code LW4292. Figure 3.7a), an extended fine tip mini pastette (104 mm length, 20 μl/drop; Alpha Laboratories, product code LW4237. Figure 3.7b), a fine tip ultra-micro pastette (51 mm length, 22 μl/drop; Alpha Laboratories, product code LW4243. Figure 3.7c) and a small bulb fine tip pastette (36 mm length, 25 μl/drop; Alpha Laboratories, product code LW4697. Figure 3.7d).

Capillary tubes of different volumes were sourced alongside various expulsion devices. To minimise the risk of whole blood clotting immediately after collection, four types of heparinised capillary tubes where sourced, two of which were glass: calibrated micropipette (127 mm length, 44.7 μl capacity; Drummond Scientific Company, product code 2-000-044-H. Figure 3.8a) and Microhaematocrit tube (40 mm length, 5 μl capacity; International Scientific Supplies, product code GC0060. Figure 3.8c). Two types of plastic capillary tubes were trialled: plasticrit plastic haematocrit tube (75 mm length, 70.27 μl capacity; Drummond Scientific Company, product code 8-000-7520-H. Figure 3.8b) and a preassembled capillary with a heparinised click clack closure

microtube (250 μl capacity; Sanguis Counting Gmbh, product code 682503. Figure 3.8d). Excluding the last preassembled tube the capillaries required a secondary method to expel the contents directly onto the test sample pad. Two types of equipment were sourced for this purpose, the first was an aspirator tube assembly (Alpha Laboratories, product code 2-000-000) where the tubing was cut short and attached to a 5 ml syringe (Figure 3.9a) and a 1 ml syringe (Figure 3.9b). The second was a simple microcap bulb dispenser (Alpha Laboratories, product code 1-000-9000. Figure 3.9c). Both systems rely on capillary action to draw up the blood, which is then dispensed by air displacement caused either by pushing down on the syringe (air drawn into before blood collection) or manually squeezing the bulb. Figure 3.10a, b & c shows each device with a different capillary tube attached.

The final blood collection device that was trialled was the Innovac ® Quick-Draw ® Capillary Whole Blood Collection System which consists of the Innovac Vacuum Handle (Innovative Med Tech, product code 15-500. Figure 3.11a), a disk filter (Innovative Med Tech, product code 15-501. Figure 3.11b), a heparin quick draw adapter (Innovative Med Tech, product code 15-980. Figure 3.11c) and a microtainer with microguard (BD, product code 365965. Figure 3.11d). The apparatus was assembled as shown in Figure 3.12. Once assembled, the battery powered vacuum handle aspirates blood droplets present on the puncture site into the container.

To make a blood drop form more easily and prevent smearing or dribbling of blood, petroleum jelly (Vaseline) was spread over the site prior to making the incision as described for human blood collection (McDermott, *et al.* 2010).

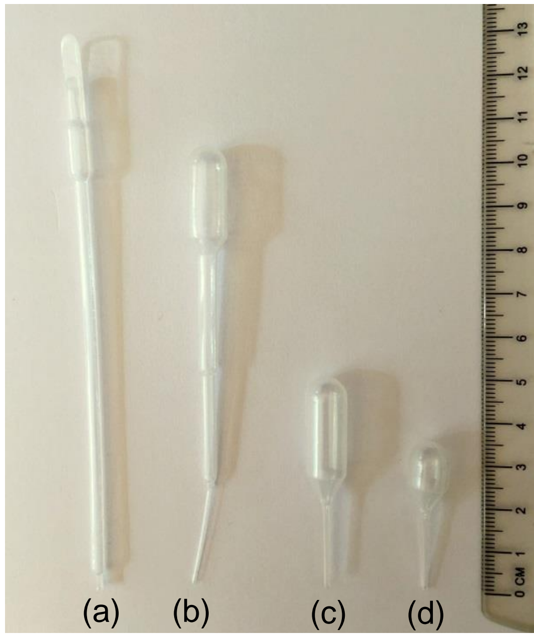

Figure 3.7. Pastettes. (a) Paddle pastette. (b) Extended fine tip mini pastette. (c) Fine tip ultra-micro pastette. (d) Small bulb fine tip pastette.

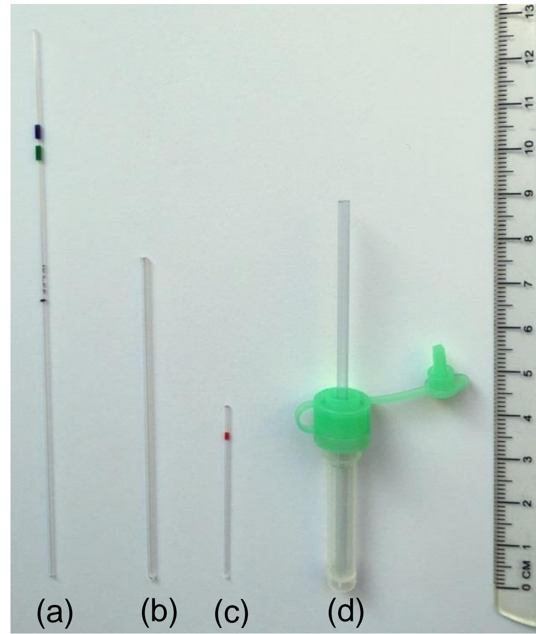

Figure 3.8. Heparin coated capillaries. (a) 44.7 µl capacity, glass. (b) 70.27 µl capacity, plastic. (c) 5 µl capacity, glass. (d) 250 µl capacity with heparinised microtube.

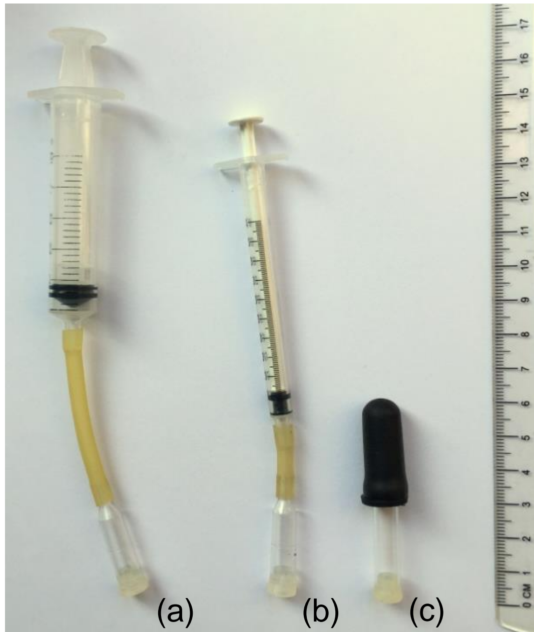

Figure 3.9. Expulsion devices. (a) Aspirator tube assembly with 5 ml syringe. (b) Aspirator tube assembly with 1 ml syringe. (c) Microcap bulb dispenser.

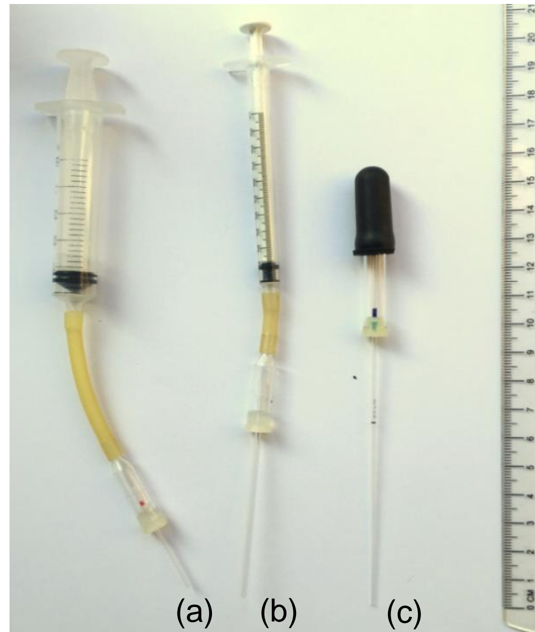

Figure 3.10. Expulsion devices with capillaries. (a) 5 ml syringe & 5 µl capillary (b) 1 ml syringe & 70.27 µl capillary. (c) Microcap bulb & 44.7 µl capillary.

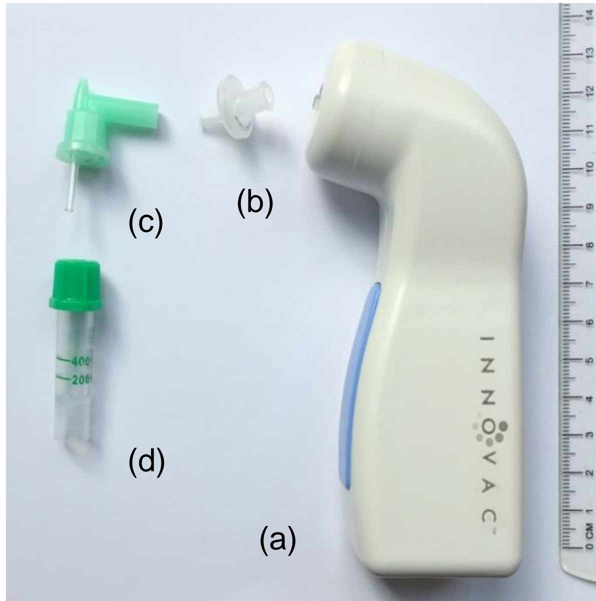

Figure 3.11. Innovac® Quick Draw® Capillary whole blood collection device. (a) Vacuum handle. (b) Disk filter. (c) Heparin quick draw adapter. (d) Microtainer.

Figure 3.12. Assembled Innovac ® Quick Draw ® Capllary whole Blood collection device.

Dual Path Platform Method

The VetTB test was carried out as per manufacturer's instructions (Chembio Diagnostic Systems Inc, 2012). In brief, collected blood was added to the round sample and buffer well one (Figure 3.13a) of the VetTB cassette followed by two drops of diluent buffer. A timer was set for five minutes, after which four drops of diluent buffer was added to the square buffer well two (Figure 3.13b). The timer was set for a further 15 minutes. After a total of 20 minutes the results were read both qualitatively (positive/negative) and quantitatively (VetTB Reader) in both the field and the laboratory. A red/pink coloured line should always be present in the control area (Figure 3.13d) if the test has been

performed correctly. In the absence of a control line after 20 minutes the test was considered to have failed. A positive test result was interpreted qualitatively as the presence of a red/pink line on either of the test lines one and two in the result window (Figure 3.13c and 3.15) and negative if there were no lines present (Figure 3.16). Results were also measured quantitatively by using the Chembio VetTB Reader (Figure 3.14), which measures the strength of each line in RLU. No negative/positive result was given by the VetTB readout as a generally applicable positive cut-off value for this test is yet to be determined.

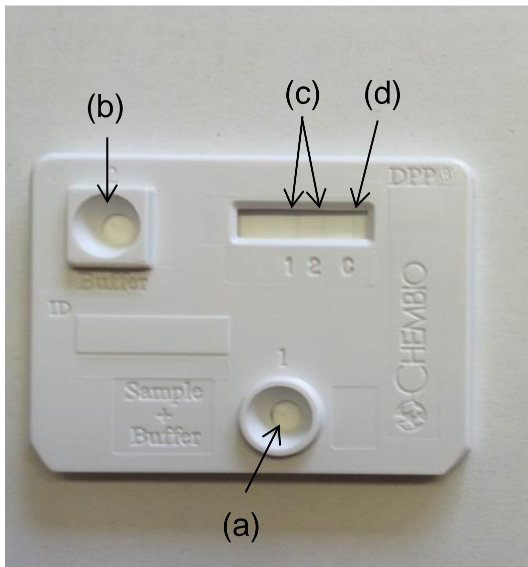

Figure 3.13. VetTB cassette used for the detection of antibodies to *M. bovis* antigen MPB83 (test line one) and ESAT6/CFP10 (test line two). (a) 10 µl sample and two drops diluent well. (b) four drops diluent well two. (c) Test line one and two. (d) Control line.

Figure 3.14. Portable Chembio VetTB reader showing the RLU of a sample tested in the field.

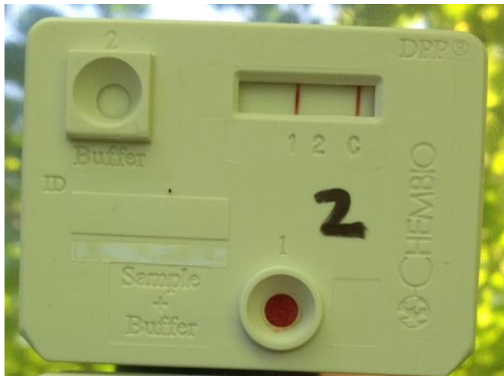

Figure 3.15. Positive VetTB result in line one with control line present. Negative VetTB result in line two. Positive/negative results assigned qualitatively.

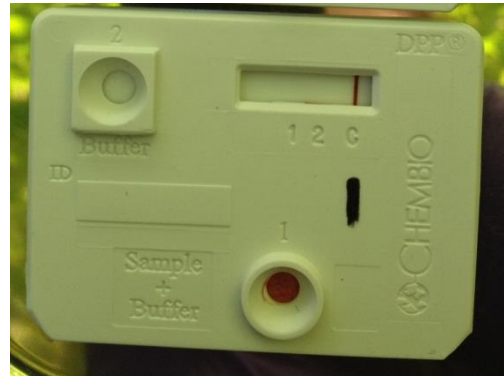

Figure 3.16. Negative VetTB result with control line present. Negative result assigned qualitatively.

Statistical Analyses

Statistical analyses were carried out to compare the VetTB test results from samples taken in the field at trap-side with those from samples taken under controlled conditions in the sampling facility and tested in the laboratory. Analyses were used to determine if there was sufficient agreement between the test procedures being carried out under different conditions. Cohen's Kappa statistic was used to test for agreement between dichotomous outcomes (positive and negative) and interpreted using the nomenclature described by Landis and Koch (1977). The association between quantitative results (RLU) was determined by Lin's Concordance Correlation Coefficient (CCC) as described by Lin (1989 and 2000) to test for agreement between the continuous measures obtained by the two methods. Qualitative and quantitative analyses

were carried out for each of the test lines (MPB83 and ESAT6/CFP10) both separately and in combination.

In addition Receiver Operator Characteristic (ROC) curves were plotted to investigate the relationship between the VetTB test results in both the laboratory and the field. A test is said to be 100% accurate against the standard it is being measured against if the ROC curve passes through the point (0,1) on the unit grid resulting in an AUC area of 1.0. The closer a ROC curve comes to this point the better its discriminating ability. A test that cannot discriminate between negative and positive corresponds to a ROC curve that follows the diagonal of the grid with an AUC area of 0.5 (DeLong *et al*, 1988). To measure the accuracy of the test, the area under the ROC curve (AUC) was calculated for each test condition and compared statistically using the methods described by DeLong *et al.* (1988). In the absence of a 'gold standard' and as the true infection status of each badger was unknown, the ROC curves produced for the VetTB test were based on the results of other imperfect routine diagnostic tests conducted on this population of badgers (Tomlinson *et al.*, 2014). Routine tests included culture of clinical samples (Gallagher and Horwill, 1997), StatPak test (Chambers *et al.*, 2008) and the IFN_{γ} (Dalley *et al.*, 2008). A study by Drewe *et al.* (2010) provided sensitivity and specificity estimates for each of these routine diagnostic tests. Culture had a low sensitivity of 8% but was highly specific at 99.8%. StatPak showed a sensitivity of 50.4% and a specificity of 96.9% and the IFN_{γ} showed a higher sensitivity of 79.9% with a specificity of 95%. ROC curves were plotted against each diagnostic test individually and in combination based on the individual badger's test history derived from the long-term study.

This included test data from previous capture events. Hence, if at any time point prior to the capture event, a positive test result was found in any of the three routine tests, then the badger was deemed positive for that test thereafter regardless of any further negative test results. An ROC curve was also plotted using the imperfect routine diagnostic tests in combination, where an overall positive outcome was determined if any of the three test results were positive.

3.4 Results

Overview

There were ten capture events involving 57 badgers during the study period. A valid VetTB test result was obtained in both the field and laboratory from 41 (72%) of these animals. The VetTB test was unavailable during the first scheduled sampling event so no tests were carried out on four badgers where blood was collected successfully. There were four unidentified individuals with results in the field only as they were not taken back to the sampling facility for subsequent sampling and identification. Blood collection was unsuccessful for eight badgers and therefore no VetTB test was carried out; of these eight, four had been released prior to any attempt to collect a sample. This included two adult animals that were released due to excessive rotation in the restraint cage, one small cub, which could not restrained due to its size and another adult that was extremely agitated. For the remaining four badgers, sample collection was unsuccessful due to insufficient blood flow.

Where blood was successfully collected, volume varied from the target of 10 µl to <5 µl (Table 3.1), with a mean of 7.4 µl. The full 10 µl of blood was collected from 22 of the 41 badgers that were restrained and provided VetTB test results for blood collected both at trap-side sampling and in the sampling facility. A useable blood sample was collected from a further 19 out of 41 animals although the quantity was less than the manufacturer's recommendation of 10 µl. As the methods were refined, sampling success increased and the target volume of 10 µl was achieved more frequently. All samples collected in the sampling facility were 10 µl in volume.

Table 3.1. Quantity of blood collected from unanaesthetised badgers at trap-side.

No. badgers	**Blood Quantity (µl)**
22	10
3	5-10
9	5
7	<5
41	Mean = 7.4 (SD = 3)

After trialling all the products described, the best performing combination for capillary blood collection was the 4 mm goldenrod animal lancet with the 40 mm length, 5 µl capacity heparinised microhaematocrit tube and microcap bulb dispenser (Figure 3.17). As a result of the capillary size, for a sample volume of 10 µl it was necessary to use two tubes.

Lancet for skin puncture

Out of the two human lancets trialled, the 2 mm blade removed from the safety casing produced a better incision compared to the 2 mm needle lancet, as the shape of the blade allowed the operator to make a slightly larger incision. However, blood flow was still insufficient and therefore the 4 mm lancet was trialled. The 6 mm goldenrod animal lancet was not trialled as the 4mm lancet was adequate at producing a large enough incision to significantly improve capillary ooze and repeatedly obtain the target volume of 10 µl.

Blood Collection Apparatus

Following successful incision, blood tended to spread outwards from the incision site without forming a droplet. This created certain difficulties when using some of the products described. The use of Vaseline to aid in droplet formation was unsuccessful as it appeared to clog the blood collection device. A thinner application of Vaseline may have prevented this from happening but this was not tested. Blood collection using the plastic pastettes was the least successful approach as in the event of a small amount of blood being collected the uptake of air with the sample resulted in the blood being sucked further up the tube and in some cases into the bulb, preventing expulsion onto the sample well of the VetTB test. The use of capillaries was more successful but depended on the diameter and length of the capillary. Both the plastic capillary and the preassembled tube were unsuccessful due to their larger diameter resulting in a poor capillary uptake. The diameter of both the 5 µl and 44.7 µl glass capillaries was the same but the larger capillary was not used in the field

as it was prone to breakages during assembly. The 5 µl capillary was the most effective at collecting a blood sample and did not break. Once collected in the capillary, the sample was expelled either using the syringe or the bulb method. Use of the syringe tended to cause the sample to spray out of the capillary whereas no spray was observed using the microcap bulb dispenser.

The Innovac Quick-Draw was tested on anaesthetised animals only. Collection of blood was unsuccessful due to spreading from the incision site with no droplet formation.

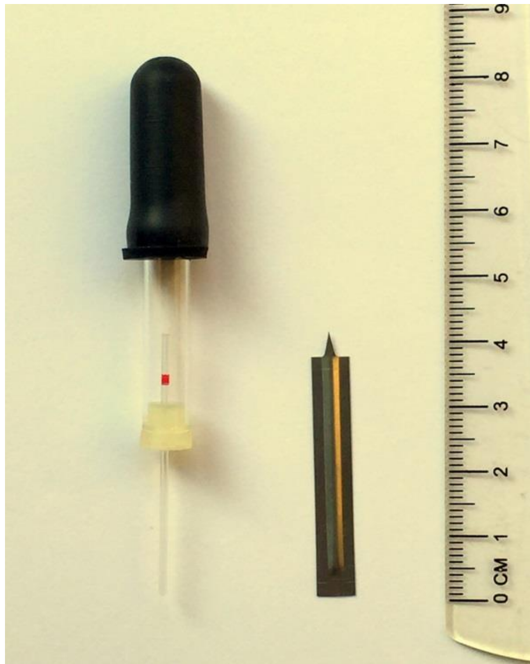

Figure 3.17. Blood collection devices of choice to successfully collect a 5 µl blood sample (two samples collected for VetTB test).

Other Observations

The time taken to deliver the blood from the capillary tube onto the VetTB test was important; after 2 minutes of collection the sample appeared to clot in the heparinised capillaries which hampered expulsion onto the sample well. This was only an issue when there was difficulty getting a second tube filled to achieve the 10 µl quantity required.

Blood flow varied between badgers with some producing better capillary ooze than others. In some instances delayed capillary ooze occurred and in other instances insufficient capillary ooze was present to take a sample.

Statistical Analyses of VetTB Test data

Qualitative Interpretation

Test Band One (MPB83)

Results showed that ten animals tested positive at trap-side compared to 14 in the laboratory where the standard protocol was used. The Cohen's Kappa index for these samples is 0.42 (Table 3.2), which indicates only moderate agreement between methods. When samples with volume <10 µl were removed, seven of the 22 remaining samples were positive at trap-side and eleven were positive in the laboratory, giving a Cohen's Kappa index of 0.46 which was still in the moderate agreement category (Table 3.3).

Table 3.2. The frequency of positive and negative results for the first test band of the VetTB test (MPB83) for all samples tested at trap-side vs laboratory (including the Cohen's Kappa index value).

	Laboratory		Total
Trap-Side	Positive	Negative	
Positive	7	3	10
Negative	7	24	31
Total	14	27	41
Kappa index	0.42		

Table 3.3. The frequency of positive and negative results for the first test band of the VetTB test (MPB83) excluding samples of <10 μl, tested at trap-side vs laboratory (including the Cohen's Kappa index value).

	Laboratory		Total
Trap-Side	Positive	Negative	
Positive	6	1	7
Negative	5	10	15
Total	11	11	22
Kappa index	0.46		

Test Band Two (ESAT6/CFP10)

Results showed that seven animals tested positive on the second test line of the VetTB test both at trap-side and in the laboratory. The Cohens Kappa index for these samples is 0.53, falling to 0.50 when samples of <10 μl were removed. In both cases there is still only moderate agreement between field and laboratory tests (Table 3.4 and 3.5).

Table 3.4. The frequency of positive and negative results for test band two of the VetTB test (ESAT6/CFP10) for samples tested at trap-side vs laboratory (including the Cohen's Kappa index value).

	Laboratory		Total
Trap-side	Positive	Negative	
Positive	5	3	8
Negative	3	30	33
Total	8	33	41
Kappa index	0.53		

Table 3.5. The frequency of positive and negative results for test band two of the VetTB test (ESAT6/CFP10) excluding samples of <10 µl, tested at trap-side vs laboratory (including the Cohen's Kappa index value).

	Laboratory		Total
Trap-side	Positive	Negative	
Positive	5	2	7
Negative	3	12	15
Total	8	14	22
Kappa index	0.50		

Test Bands Combined

When combining the results of the VetTB test for test lines one and two, 16 animals tested at trap-side had a positive result with 17 also testing positive in the laboratory. A Cohen's Kappa index value of 0.44 was calculated for all samples and was unchanged when samples of <10 µl were removed, indicating moderate agreement (Table 3.6 and 3.7).

Table 3.6. The frequency of positive and negative results for the VetTB test when both test bands were combined for samples tested at trap-side vs laboratory (including the Cohen's Kappa index value).

	Laboratory		Total
Trap-side	Positive	Negative	
Positive	11	5	16
Negative	6	19	25
Total	17	24	41
Kappa index	0.44		

Table 3.7. The frequency of positive and negative results for the VetTB test when both test bands were combined but excluding samples of <10 µl, tested at trap-side vs laboratory (including the Cohen's Kappa index value).

	Laboratory		Total
Trap-side	Positive	Negative	
Positive	10	2	12
Negative	4	6	10
Total	14	8	22
Kappa index	0.44		

Quantitative Interpretation

VetTB Line One (MPB83)

Relative light units ranged from 22.34-6425 (median 60.87) for trap-side tests and 7.41-6611 (median 67.54) for laboratory tests. When results from the two approaches were compared, Lin's concordance correlation coefficient (CCC) varied depending on the quantity of blood in the field sample. Samples of 10 µl showed the best agreement with a CCC of 0.79 reducing to 0.24 for field

samples of <10µl. All samples combined resulted in a CCC value of 0.78 (Table 3.8 and Figure 3.18).

VetTB Line Two ESAT6/CFP10

RLU ranged from 0.00-226.2 (median 0.00) for trap-side tests and 0.00-308.5 (median 1.87) for laboratory tests. When results from the two approaches were compared, Lin's concordance correlation coefficient varied depending on the quantity of blood sample used. Samples of 10 µl showed the best agreement with a CCC of 0.51 reducing to -0.10 for samples of <10µl. All samples combined resulted in a CCC value of 0.43 (Table 3.8 and Figure 3.18).

Table 3.8. Lin's concordance correlation coefficient (CCC) values showing 95% confidence intervals for VetTB test lines one and two.

Line 1 MPB83	**CCC**	**95% CI**
All Samples	0.78	0.65-0.87
10 µl volume	0.79	0.58-0.90
<10 µl volume	0.24	0.15-0.33
Line 2 ESAT6/CFP10		
All Samples	0.43	0.17 - 0.64
10 µl volume	0.51	0.14 - 0.76
<10 µl volume	-0.10	-0.38 - -0.20

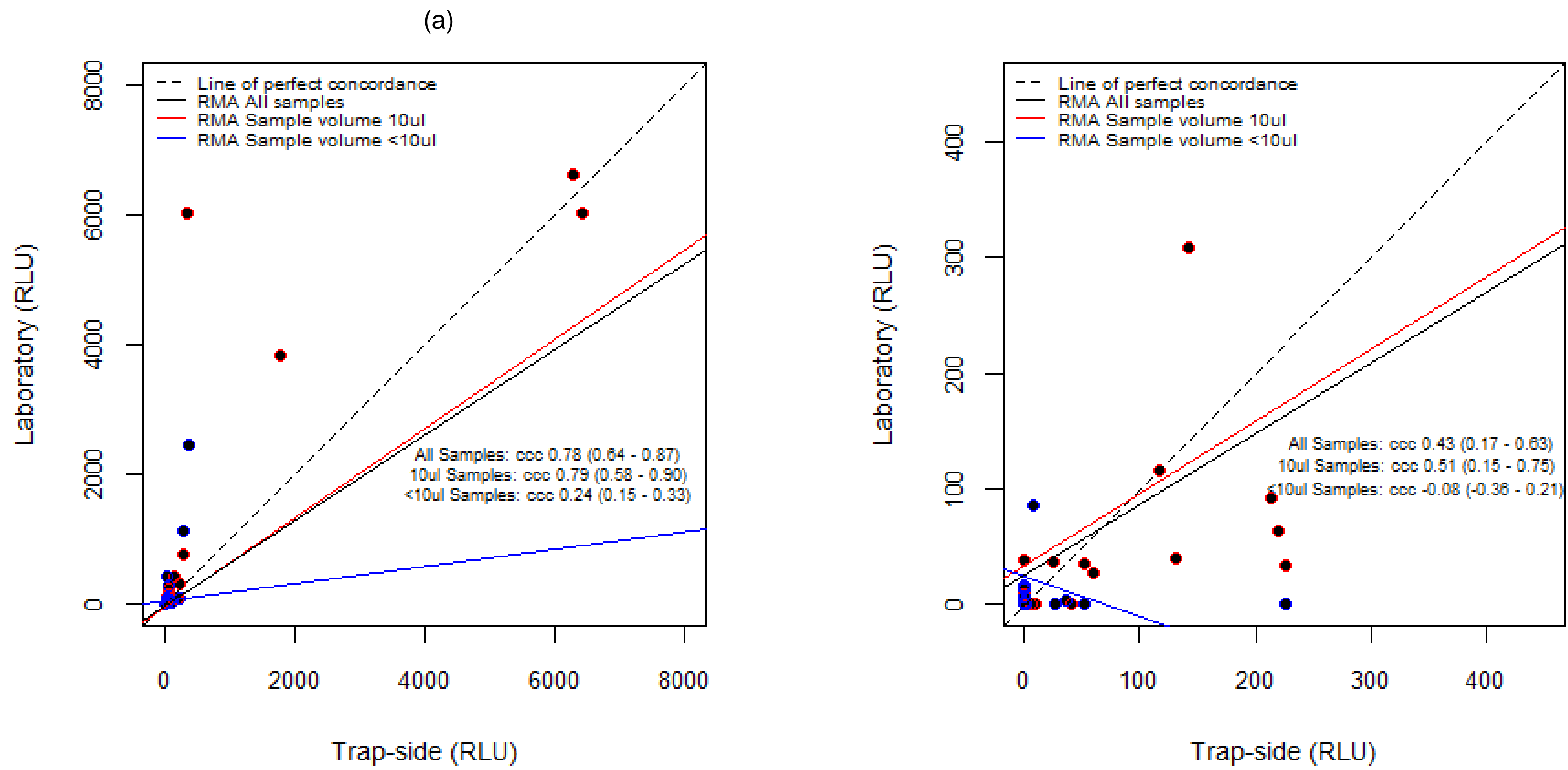

Figure 3.18. Scatter plots showing concordance of RLU between samples tested at trap-side and in the laboratory a) VetTB test line one (MPB83) b) VetTB test line two (ESAT6/CFP10).

ROC Analyses

ROC analyses for VetTB test line two shows mostly low AUC values relative to the reference tests, apart from those tested at trap-side against culture results (Table 3.9). ROC analyses for VetTB test line one showed that tests carried out in the laboratory were more accurate against the reference tests StatPak, IFN_{γ} and tests combined compared to those carried out at trap-side. When culture was used as the reference test, the opposite was observed where VetTB results from field tests were more accurate than those tested in the laboratory (Table 3.9).

Culture ROC Analyses

ROC analyses carried out for culture results were based on 34 negative cases and seven positive cases. An AUC value was calculated separately for samples tested at trap-side and in the laboratory for both test lines. No significant difference was found between curves for either test line (Table 3.9. Figure 3.19).

StatPak ROC Analyses

ROC analyses carried out for StatPak results were based on 21 negative cases and 20 positive cases. An AUC value was calculated separately for samples tested at trap-side and in the laboratory for both test lines. No significant difference was found between curves for either test line (Table 3.9. Figure 3.20).

IFN_{γ} ROC Analyses

ROC analyses carried out for IFN_{γ} results were based on 26 negative cases and 15 positive cases. An AUC value was calculated separately for samples tested at trap-side and in the laboratory for both test lines. A significant difference was found between AUC values for trap-side and laboratory tests for VetTB test line one ($p<0.04$) and test line two ($p<0.018$) with the laboratory tests showing better agreement than field tests (Table 3.9. Figure 3.21).

Combined Tests ROC Analyses

ROC analyses carried out for the combined test results were based on 18 negative cases and 23 positive cases. An AUC value was calculated separately for samples tested at trap-side and in the laboratory for both test lines. No significant difference was found between curves for either test line (Table 3.9. Figure 3.22).

Table 3.9. Statistical analyses of AUC values generated from samples tested by VetTB test at trap-side and in the laboratory, against three imperfect diagnostic tests, individually and in combination. Values shown in bold represent a significant difference between AUC values between the two test conditions.

Line 1 MPB83	**Culture DS**	**Stat Pak DS**	**IFN$_\gamma$ DS**	**Tests Combined**
Trap-side AUC 95% CI	0.76 0.53-0.99	0.74 0.59-0.89	0.83 0.70-0.96	0.75 0.60-0.90
Laboratory AUC 95% CI	0.70 0.40-1	0.85 0.73-0.98	0.96 0.91-1	0.87 0.76-0.98
Z-Value	0.74	1.50	2.05	1.55
P-Value	0.46	0.13	**0.04**	0.12
Line 2 ESAT6/CFP10				
Trap-side AUC 95% CI	0.73 0.61-0.85	0.51 0.34-0.68	0.65 0.49-0.81	0.5 0.32-0.67
Laboratory AUC 95% CI	0.55 0.34-0.75	0.59 0.41-0.76	0.45 0.26-0.64	0.61 0.44-0.78
Z-Value	1.49	-0.53	-2.37	-0.78
P-Value	0.13	0.59	**0.018**	0.44

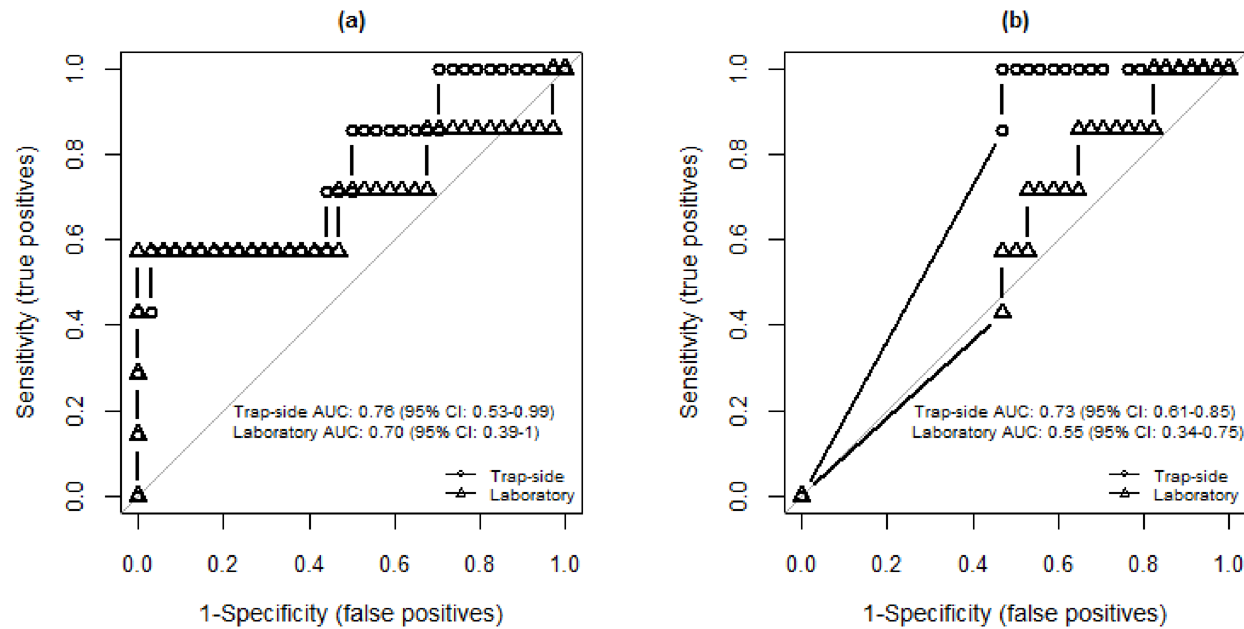

Figure 3.19. ROC curve analyses for VetTB test performance in relation to the culture disease status of badgers from samples tested at both trap-side and in the laboratory including AUC values with 95% CI. Each point on the curve represents a different RLU value for the test and is plotted against the sensitivity and specificity corresponding to that RLU value, based on the culture disease status of 41 badgers used for testing. (a) Test line one (MPB83) and (b) test line two (ESAT6/CFP10).

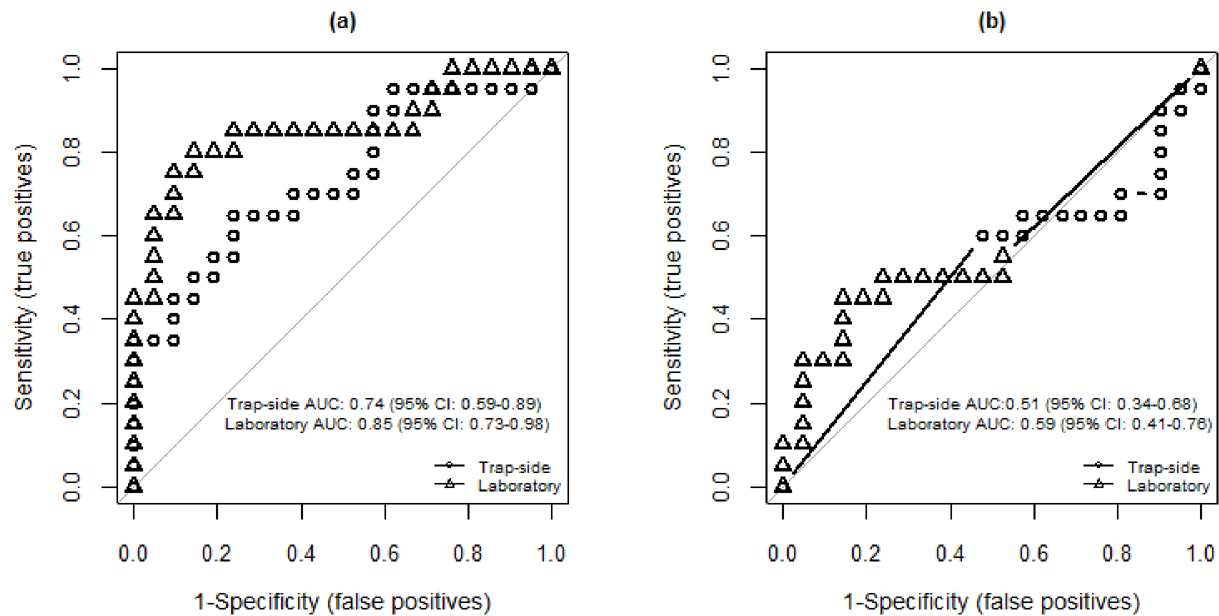

Figure 3.20. ROC curve analyses for VetTB test performance in relation to the StatPak disease status of badgers from samples tested at both trap-side and in the laboratory including AUC values with 95% CI. Each point on the curve represents a different RLU value for the test and is plotted against the sensitivity and specificity corresponding to that RLU value, based on the StatPak disease status of 41 badgers used for testing (a) test line one (MPB83) and (b) test line two (ESAT6/CFP10).

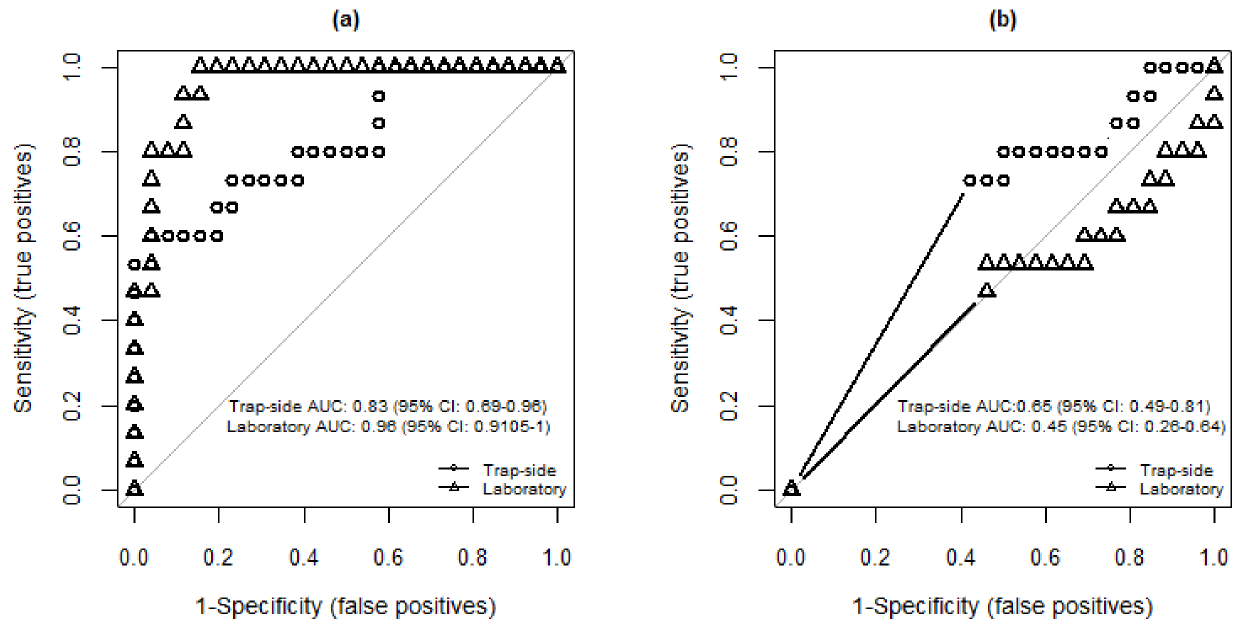

Figure 3.21. ROC curve analyses for VetTB test performance in relation to the IFN_{γ} disease status of badgers from samples tested at both trap-side and in the laboratory including AUC values with 95% CI. Each point on the curve represents a different RLU value for the test and is plotted against the sensitivity and specificity corresponding to that RLU value, based on the IFN_{γ} disease status of 41 badgers used for testing (a) test line one (MPB83) and (b) test line two (ESAT6/CFP10).

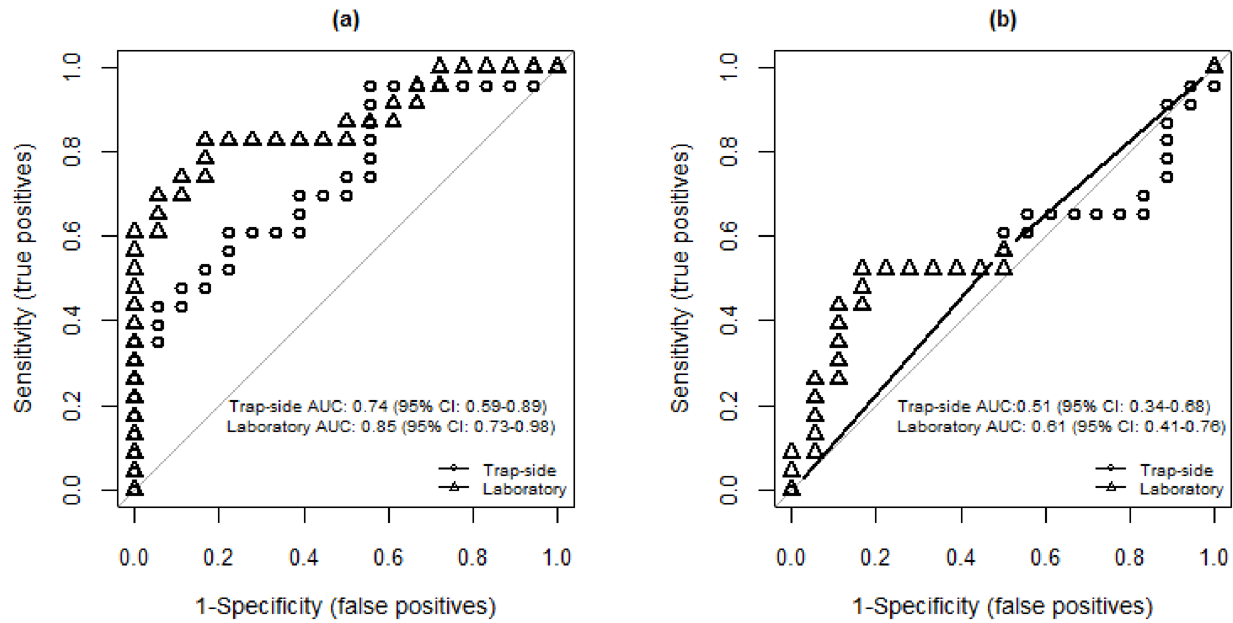

Figure 3.22. ROC curve analyses for VetTB test performance in relation to combined diagnostic test disease status of badgers from samples tested at both trap-side and in the laboratory including AUC values with 95% CI. Each point on the curve represents a different RLU value for the test and is plotted against the sensitivity and specificity corresponding to that RLU value, based on the combined diagnostic test disease status of 41 badgers used for testing (a) test line one (MPB83) and (b) test line two (ESAT6/CFP10).

3.5 Discussion

The aim of this study was to develop a method of collecting a viable capillary blood sample from an unanaesthetised badger in the field and to evaluate its use to diagnose bTB infection using a rapid trap-side test for use in the field. Development of a repeatable and cheap method to achieve this may aid the implementation of future policy for TB control programmes such as a test and vaccinate/remove option.

With use of a novel restraint cage which allowed safe access to the badger's hind leg the development of a method to collect a target blood volume of 10 µl from unanaesthetised badgers at trap-side was achieved during the course of this study. Continuous refinement of the method meant the target blood volume was reached with increasing reliability. A higher success rate was particularly apparent when using the 4 mm goldenrod animal lancet, 5 µl microhaematocrit tubes and microcap bulb (Figure 3.17).

In the four cases where blood collection was not attempted, cage design appeared to be the limiting factor. Excessive rotation by the badger prevented the operator from gaining access to a hind leg in two adults. The cage was not appropriate for restraining small cubs. Further cage improvements may be required to reduce the likelihood of this re-occurring. A separate smaller cage design for use with cubs could be explored.

When blood collection was attempted there were several obstacles apparent. On four occasions insufficient blood flow to the incision site prevented collection of a suitable sample. It was noted that this occurred on mornings that were particularly cold, although air temperature was not routinely recorded as part of

the study. Literature regarding blood collection in humans describes rubbing the area prior to incision and use of heat pads to increase the rate of blood flow to the target site and allow more blood to be collected before bleeding ceases. (Ellison *et al.*, 2002 and Edelbroek *et al.*, 2009). Use of heat pads could be an option to encourage blood flow to the area especially if sampling in colder weather, although the badger's welfare would need to be taken into consideration as this could mean a longer restraint time potentially causing additional stress to the badger.

Another observation was the difficulty of getting blood from badgers that appeared dehydrated or in bad condition possibly due to disease. Two of the four badgers where no blood sample was achieved had a positive *M. bovis* culture result. Anecdotal evidence of badgers sampled under anaesthesia suggests increased difficulty in collecting blood when the animal is in very advance stages of disease, although this has not previously been reported in the literature.

The limited volume of the microhaematocrit tube meant that to achieve a target blood quantity of 10 µl two separate tubes were required. This was not ideal as the blood had a tendency to clot in the heparinised tube if the second sample was not achieved in good time. Clotting of blood samples hindered expulsion onto the test area and reduced the quantity of blood available.

Current diagnosis of *M. bovis* infection in live badgers relies on samples collected under anaesthesia which is labour-intensive, time-consuming and expensive. In addition, most of the diagnostic tests currently available take too long to provide trap-side results and require specialist laboratory facilities and

equipment. Both culture of clinical samples and the IFN_{γ} test also rely on sample types and quantities that would be difficult if not impossible to collect from an unanaesthetised badger. Serological tests such as the VetTB test that detects antibodies to *M. bovis* antigens such as MPB83 and ESAT6/CFP10 are a better option. This test requires only a 10 µl sample, can be performed anywhere and produces a result in no more than 20 minutes. Successfully collecting blood from 41 unanaesthetised badgers meant that the VetTB test could be performed directly in the field and results compared to tests carried out under controlled conditions in the laboratory.

Analyses revealed there was only a moderate agreement between test conditions regardless whether the full 10 µl target sample was achieved in the field or not. The stochastic conditions present in the field may affect the performance of the test. Samples are more likely to be contaminated when taken in the field due to the surrounding environmental conditions. Dilution of the sample due to contaminants may result in a lower concentration of antibodies and it is unknown what direct effect the contaminants may have on the VetTB test. Another consideration is regarding the VetTB method which requires the test to be carried out at a temperature of 18 to 30°C on a flat surface and interpreted in well-lit conditions (Chembio Diagnostic Systems, 2012) as found in the laboratory. When conducting tests at trap-side it is unlikely that the temperature would have been 18°C or higher especially during the colder months (January) where temperatures where likely <10°C. It is unknown what effect this may have had on the test and without a record of the temperature this aspect could not be explored in detail. Due to the nature of the terrain finding a flat level surface to carry out the test was difficult potentially

affecting the flow of sample and diluent along the cassette. A wooded overhead canopy coupled with low light levels in the early mornings could also affect test interpretation.

Regardless of the low level of agreement between methods it was apparent when looking at the related RLU values (Figure 19) that any animals with a strong positive response were picked out in both the field and laboratory and therefore animals at more advanced stages of disease are likely to be detected in both scenarios. This would imply that the VetTB test could be used as a simple tool to detect badgers at advanced stages of disease. When determining the accuracy of the two test conditions against other imperfect diagnostic tests a significant difference was only found when using the IFN_{γ} as reference. The IFN_{γ} is more sensitive than both the culture and the StatPak therefore more indicative of the badger's true infection status compared to the other less sensitive tests. With this is mind, results show that tests carried out in the controlled conditions of the laboratory under the manufacturers recommendations are significantly more accurate than those carried out in the stochastic conditions of the field.

In conclusion we have developed a technique for collecting capillary blood from the hind paw of an unanaesthetised badger and applying it to a serological test in the field. With only a moderate agreement between tests carried out in the field and laboratory, further ways to replicate laboratory conditions should be considered. A way to create a level surface within a temperature controlled environment with an overhead light may help enhance test accuracy. In addition improvements in the quality of blood sample collected may be achieved by

sourcing a 10 µl volume heparinised capillary tube with the same bore width as the 5 µl one successfully trialled. This would prevent the need for two samples to be taken and therefore reducing the likelihood of the blood clotting. Warming the target area prior to incision may help increase blood flow making the blood more easily accessible for collection. The sensitivity and specificity of the VetTB test needs to be evaluated to determine if it would be sufficiently sensitive for routine TB surveillance. The sensitivity may also be explored against animals with more severe TB as a higher sensitivity in these animals would mean that the VetTB test could be used to remove badgers at more advanced stages of disease that are more likely infectious.

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CHAPTER 4 General Discussion

Diagnosing disease in wildlife can be more challenging than in domestic animals and humans. Many routine diagnostic tests have been developed for use in detecting or confirming disease in domestic species and have not been validated for use in wildlife. Wild animals are subject to a wide range of genetic and environmental factors many of which may influence their immune response such as genetic variability, history of infection (immune memory, current infections), physiological condition (e.g. sex, age, reproductive state and history), resource availability (e.g. diet), abiotic conditions (e.g. seasonality and temperature) and co-evolutionary history between host and pathogen (Pederson and Babayan, 2011). These additional pressures may affect the immune system in different ways and within a group of animals, individuals will vary in their immune responses, some may have a strong response and others weak. In Chapter 2 I provide evidence of between-individual variation in the cell-mediated immune responses of free-living badgers to a range of intrinsic factors. Results showed both augmentation and suppression of the general immune response. My analysis also revealed factors that augment the specific *M. bovis* response. Clearly, incorporation of knowledge of these intrinsic factors into diagnostic determination of the bTB infection status has potential to improve test performance, by controlling for variation in test outcomes that does not arise from infection status.

In the last few decades increased awareness of the role of wild animals in the maintenance and spread of important zoonotic infections means that research has been directed into development of specific diagnostic tests aimed at wildlife.

Bovine tuberculosis (bTB) is one such example where a variety of different tests are available for badger bTB diagnostics. Diagnostic tests are used to determine whether an individual is disease free, infected or has previously been exposed to a specific pathogen. Tests that detect the pathogen, such as culture and PCR show similar results in both wildlife and domestic animals (Bengis *et al.*, 2002). However due to intermittent shedding of bacteria and low test sensitivity of culture (8%) there is a high chance that infected individuals are missed. Culture also takes several weeks for a result and would be impractical for use in a bTB control programme. Indirect tests that detect exposure to a specific pathogen usually measure some aspect of the animal's immune response and are more variable between wild and domestic animals. The gamma interferon test is highly sensitive and specific but labour intensive and requires specialist laboratory facilities. This test would therefore not be a financially viable or practical option for use in a disease control programme. Serological tests such as the StatPak and VetTB assays are a much more attractive option with regard to practicality and cost, the StatPak however only has a moderate sensitivity and it would be questionable as to whether it would be sufficient to use in a control programme and has now been discontinued by the manufacturer. The VetTB assay to date has not yet been fully evaluated. The sensitivity and specificity of a test is important especially when there is a possibility of use within a disease control programme. Low sensitivity means that there is an increased likelihood of false negative results and infected animals may remain in the population. Low specificity, on the other hand, means that there is an increased likelihood of a false positive result potentially

causing unnecessary removal of non-infected animals, which has further welfare and cost implications.

In Chapter 3 I have shown the importance of environmental conditions and blood quality on test performance. Diagnostic tests are usually developed in the controlled conditions of a laboratory, as indicated in the manufacturer's methods, and are implemented by specialist personnel. These conditions are not easily replicated in the field and in some cases unrealistic. Blood collection from an unanaesthetised animal provides more challenges and collecting an aseptic blood sample as you would in an animal under anaesthesia is unlikely. Although the effects of these factors on test performance aren't readily distinguished, we have shown that when using the VetTB assay in the field there is a negative effect on test accuracy. To try and improve accuracy it is important that the environmental conditions are replicated as much as possible to reflect the laboratory when testing in the field. The results of the diagnostic assay used in this study were recorded both qualitatively as a binary outcome (negative/positive) and quantitatively as reflectance light units (RLU). Due to the difference found in test accuracy, use of the RLU may be a preferred option as a lower cut-off value could be applied to samples tested in the field. This highlights the wider advantage of diagnostic tests having a quantitative output, compared to a visual positive/negative result based on an operator's opinion. Although the VetTB assay showed a reduction in accuracy when used under field conditions the potential for its use as a tool to detect individuals at more advanced stages of diseases means that it holds potential as a tool for use in bTB control programmes where removing animals that are likely to shed *M. bovis* is a desired option.

Surveillance of wild animal diseases that spread to domestic animals and humans is important, and in the case of bTB, essential to aid in the control of disease spreading to livestock. The development of practical, sensitive and specific diagnostic tests for use in badgers would enable a better understanding of the geographical spread of bTB infection in badgers. A non-invasive blood sampling device used alongside a test that identifies an infected badger could mean that future interventions are targeted at individual badgers or setts, rather than the whole population (DEFRA, 2015). In principle, removal of infectious individuals reduces transmission of disease within and between-species and limits environmental contamination. In addition the removal of all animals from a sett designated as infected by the presence of one or more infected animals could further reduce the likelihood of transmission. However previous culling trials have identified social perturbation amongst badger populations resulting in increased transmission between badgers and from badgers to cattle in adjacent areas (Woodroffe *et al*., 2006). The removal of fewer animals might be expected to reduce the level of disruption caused by culling and hence these negative epidemiological effects (Bielby *et al*., 2014).

The development of strategies to control diseases shared with wildlife are aimed at reducing pathogen transmission to domestic animals and humans. In all disease control programmes targeting wild animals there can be a strong public opposition especially when non-targeted culling is introduced. At present, without the appropriate diagnostic tools and difficulties associated with handling wild animals, the only measure available to remove infected animals from a population is by controlling it through culling. While the only option for reducing the availability of susceptible animals is injecting vaccine. In the UK there is a

significant public opposition to culling badgers for the control of bTB in cattle (White *et al*., 2000) and therefore a non-lethal or targeted cull option may be more socially and politically attractive such as vaccination or a test vaccinate/remove option (Bielby *et al*., 2014).

The recognition of bTB as a global threat at the wildlife-livestock-human interface has meant that badgers alongside other wildlife species such as the brushtail possum (*Trichosurus vulpecula*) in New Zealand (Coleman and Cooke, 2001), white-tailed deer (*Odocoileus virginianus*) in the United States (Schmitt *et al*., 1997) and European wild boar (*Sus scrofa*) in Spain (Naranjo *et* al., 2008) have been the focus of much research with diagnostic tests developed specifically to diagnose bTB in these species. This however is not the case for many other emerging infectious diseases where the important role that wildlife plays in the dynamics of disease transmission has been increasingly recognised.

With 60% of emerging infectious disease events in humans caused by zoonotic pathogens and 72% of these identified from a wildlife origin, diseases in wildlife represent one of the most significant growing threats to global health (Jones *et al*., 2008). With this in mind including the impact of wildlife disease on agricultural production, biodiversity, animal welfare and the economy it is becoming increasingly important to gain a deeper understanding of their role when considering disease management. Where knowledge of disease management is limited in species or diseases that have received less attention, more research is required through surveillance and descriptive studies (Gortázar *et al*., 2007). The availability of a suitable diagnostic test to diagnose

diseases in wildlife is becoming increasingly important especially when considering future disease management recommendations.

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