The effects of oxidative stress and innate immunity on European badger (*Meles meles*) life-history dynamics

Kirstin Louise Bilham

Lincoln College

University of Oxford

Thesis submitted for the degree of *Doctor of Philosophy*

Trinity Term, 2016

Preface

Abstract

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Wild animals are faced with many stressors from starvation to infections, which will affect individual survival and population dynamics. Free radicals and reactive oxygen species are involved in many of these processes, from cell signalling and immunity, to potentially being a causal factor in ageing. Oxidative stress is posited as a major factor in population dynamics, ultimately driving the evolution of life-history traits in wild species, acting as the 'currency' through which various trade-offs operate. In this thesis, I present a series of studies on the Wytham population of European badgers (*Meles meles*) focussed on dissecting the effects of host responses to stressors and pathogen recognition on life-history success.

The first part of this thesis investigates intrinsic and extrinsic factors affecting oxidative stress and antioxidant defences, linked to population-dynamic effects and life-history traits. I model the crucial intrinsic (development, ageing, reproduction) and one important extrinsic (weather conditions) factors affecting oxidative stress levels in a wild mammal, in relation to survival and reproductive investment. Benefitting from a long term study with marked individuals of a known age, I found that cubs trade-off the development of antioxidant defences against growth, and that juvenile survival is dependent on levels of oxidative damage. These factors are interactive with prevailing yearly weather conditions; investment in antioxidant defences has the greatest survival benefit for young cubs in harsh years (dry, cold spring). I also show that weather affects age-classes in different ways, but I did not find any link between higher oxidative damage and ageing, even following individual responses.

The second major focus for the thesis is to explore the immune capability of badgers, focussing on macrophage function and the initial recognition of pathogens. Wildlife immunology represents a "neglected area" of ecology that has potential for large impacts in terms of conservation and disease management. Indeed, wildlife can be a reservoir for infections that affect humans (zoonotic infections) and their livestock. The badger is a classic example of this effect being considered an important reservoir for *Mycobacterium bovis*, the cause of bovine tuberculosis (bTB). I employed molecular immunology methodologies to develop a methodological tool box to assess immune responses in badgers, which are then employed to investigate the response of blood monocyte-derived macrophages to microbial agonists that stimulate Toll-like receptors. The major findings are that badger macrophages fail to produce nitric oxide or meaningfully upregulate inducible nitric oxide synthase mRNA after exposure to TLR agonists, bacterial lysates and / or recombinant badger Interferon gamma. The TLR system is demonstrated to be largely intact in badger macrophages since exposure to TLR agonists did induce upregulation of cytokine mRNA. The only agonists that stimulated very low responses are those that target TLR9, but this was due to very low levels of expression of TLR9 mRNA. Both nitric oxide and TLR9 are implicated in responses to bTB and these deficiencies would significantly impact on the susceptibility of badgers to infection.

There are numerous wider implications of my work: My thesis foremost highlights the importance of taking an integrated approach to eco-physiology and eco-immunology, as these processes are heavily intertwined. The weather correlates of oxidative stress not only highlight the potential vulnerability of all species to human induced rapid environmental change, but provide a stark warning especially for less resilient specialists. The immunology work confirms the importance of carefully analysing immune responses of wildlife species, especially in the context of designing effective species management strategies for diseases such as bTB, providing indicators of why a species may be susceptible to infection and indicating potential ways to improve vaccination.

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Declaration

I declare that the work in this thesis was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes, and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, all work is my own. Work done in collaboration with, or with the assistance of others, is indicated as such. Chapter 7 is currently submitted to The Journal of Immunology, with all authors acknowledged.

Signed:…….. Date: ……..

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Abbreviations

AIC	Akaike information criterion
ANCOVA	Analysis of co-variance
AO	Antioxidant
AOX	Antioxidant capacity
APC	Antigen-preseting cell
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guerin
BCI	Body condition index
bdIFNγ	Recombinant badger interferon gamma
bdMφ	Badger macrophage
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
bTB	Bovine Tuberculosis
cDNA	Complementary DNA
CI	Confidence intervals
COE	Carry-over effects
ConA	Concanavalin A
CpG	Cytosine-phosphate-guanosine
DAMP	Damage associated molecular patterns
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
Ds	Double stranded
eNOS	Epithelial NOS
FCS	Foetal calf serum
FGN	Flagellin
FRTA	Free radical theory of ageing
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPDH	Glycerol – 3-phosphate dehydrogenase
GH	Growth hormone
GOI	Gene of interest
GPS	Global positioning system
HIREC	Human induced rapid environmental change
HKLM	Heat killed *Lysteria monocytogenes*
HKTB	Heat killed *Mycobacterium tuberculosis*
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPCC	Intergovernmental Panel on Climate Change
IPTG	Isopropyl beta-D-1-thiogalactopyranoside
KRL	Red blood cell killing assay
LAM	Lipoarabinomannan from *M. smegmatis*
LP	Lipid peroxidation

LPS	Lipopolysaccharide
LRS	Lifetime reproductive success
mDAMP	mitochondrial DAMP
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MΦ	Macrophage
NLR	nucleotide-binding oligomerization domain-like receptors
nNOS	Neuronal NOS
NO	Nitric Oxide
ODBA	Overall dynamic body acceleration
ODN	Oligodeoxynucleotide
OS	Oxidative stress
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PER	Peroxidase
Poly (I:C)	Polyriboinosinic:polyribocytidylic acid
PRR	Pattern Recognition Receptor
DSH	Disposable soma hypothesis
PUFA	Poly-unsaturated fatty acids
qRT-PCR	Qyantitative reverse transcription polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RBC	Red blood cell
RFID	Radio-frequency identification
RNA	Ribonucleic acid
RNase	Ribonuclease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTA	Road traffic accident
SNP	Single nucleotide polymorphism
Ss	Single stranded
TB	Tuberculosis
TBARS	Thiobarbituic acid reactive species
Th1	T-helper 1
TIR	Toll/IL1R homology domain
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
UA	Uric acid
WBC	White blood cell
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1: **General introduction**

The life-history strategies of wild animals involve adaptive responses to a variety of physical and biotic factors. The first include weather, habitat conditions and food availability, while the latter comprise of disease, intra-specific competition, mate selection and reproduction, and senescence (Munns Jr 2006, Acevedo-Whitehouse and Duffus 2009). In turn, life-history optimality determines fitness, and thus is fundamental to evolutionary ecology and conservation applications (Zera and Harshman 2001, Mangel 2008).

Metabolic stress, however, causes reactive oxygen species (ROS) to be generated, which can cause cellular damage. The ability of an individual (or species) to mitigate this 'oxidative stress' (OS; Sies 1997) through its antioxidant capacity is crucial, but costly, and must be traded-off against investment in other physiological and immunological processes, affecting optimisation of various life-history strategies (Sheldon and Verhulst 1996, Monaghan *et al.* 2009).

In terms of evolutionary fitness, life-history traits, such as lifetime reproductive success (LRS), somatic conservation and realised lifespan are generally inter-dependent and therefore cannot evolve independently (Stearns 1992). This leads to energetic budget allocation trade-offs (Stearns 1983, 1989, Perrin and Sibly 1993, Pörtner *et al.* 2006, Moore and Hopkins 2009) between investment in reproduction, somatic conservation and longevity. In this context, immune function is a key factor as it mediates survival and LRS by fighting off disease and pathogens and developing immune memory to infections (Zera and Harshman 2001, Witter *et al.* 2012, Korfel *et al.* 2015).

This thesis will investigate how physiology, immunity and environmental conditions influence the ecology of a versatile, generalist-omnivorous Carnivore species, the European badger (*Meles meles*). Badgers are a highly suitable study species due to their social organisation and high population density in the UK (Stopka and Johnson 2000) as well as the wealth of existing knowledge (Macdonald and Newman 2002, Dugdale *et al.* 2011b, Macdonald and Feber 2015; see additional details in Chapter 2). Their easy trappability allows for repeat individual sampling across seasonal trappings. Their comparatively large size for UK mammals permits sample volumes (especially of blood) to be taken that are sufficient for multiple experiments and analyses.

An inter-twined pair of factors that are relevant here, are those of innate immunity and oxidative stress – too often overlooked in comparison to more readily observed ecological correlates, such as food supply and habitat suitability. These are two particularly important and linked physiological aspects, which influence their response to pathogens as well as their ability to deal with metabolic stressors. Nevertheless, there is currently little existing work on physiology in badgers, and especially OS and immunology (but see Lesellier *et al.* 2006, Lesellier *et al.* 2009b, Bilham *et al.* 2013, Chambers *et al.* 2014).

With this thesis, I provide one of the first longitudinal studies of oxidative stress in a wild mammal, investigating various aspects of OS such as antioxidant molecules and enzymes, as well as levels of oxidative damage. In addition, I also characterise the early detection of pathogens via Toll-Like Receptors in badger macrophages.

Specifically, this thesis aims to:

- Provide longitudinal data on oxidative stress in a wild mammal (Chapter 4) and investigate if OS levels vary with age (Chapter 4).
- Establish if reproduction in females causes changes in OS physiology (Chapter 4).
- Investigate if extrinsic factors (e.g. weather) affect OS levels in badgers (Chapter 5)
- Identify and characterise some of the genes involved in immunological processes in badgers (Chapter 6)
- Investigate responses to specific TLR agonists in badger macrophages (Chapter 7)
- Characterise the immunological response of badger macrophages and infection with TB (Chapter 7)

In order to investigate the above mentioned questions, I will present the necessary theoretical framework regarding oxidative stress and immunology.

1.1 **Free radicals and oxidative stress**

Free radicals are molecules with unpaired electrons, prone to reactivity - also termed reactive oxygen species (ROS; Harman 1956, Halliwell 2012) that can cause cumulative molecular damage to cells and organisms (Harman 1956, Beckman and Ames 1998, Finkel and Holbrook 2000, Nemoto and Finkel 2004, Selman *et al.* 2012). During cellular respiration, the electron transport chain transfers electrons from a donor (the reduced form of nicotinamide adenine dinucleotide, NADH) to an acceptor (O_2), with the transfer of a proton through the mitochondrion membrane, creating a proton gradient (Fig. 1.1). This proton gradient is used to produce energy in the form of adenosine triphosphate (ATP; Alberts 2008, Brown 1992). ROS are produced mainly as by-products of this cellular respiration (metabolism) where electrons can leak before reaching the end of the electron transport chain (Ott *et al.* 2007), which then react with

oxygen molecules, generating superoxide anions (O2−; Turrens 1997). In turn, superoxide is modified to form molecules of varying reactivity (e.g., hydroxyl radicals - •HO , hydrogen peroxide - H_2O_2; Sies 2014-, singlet oxygen - $^1O_2$; Bayir 2005). ROS can also be generated through exposure to pollution and radiation (Poljšak and Fink 2014).

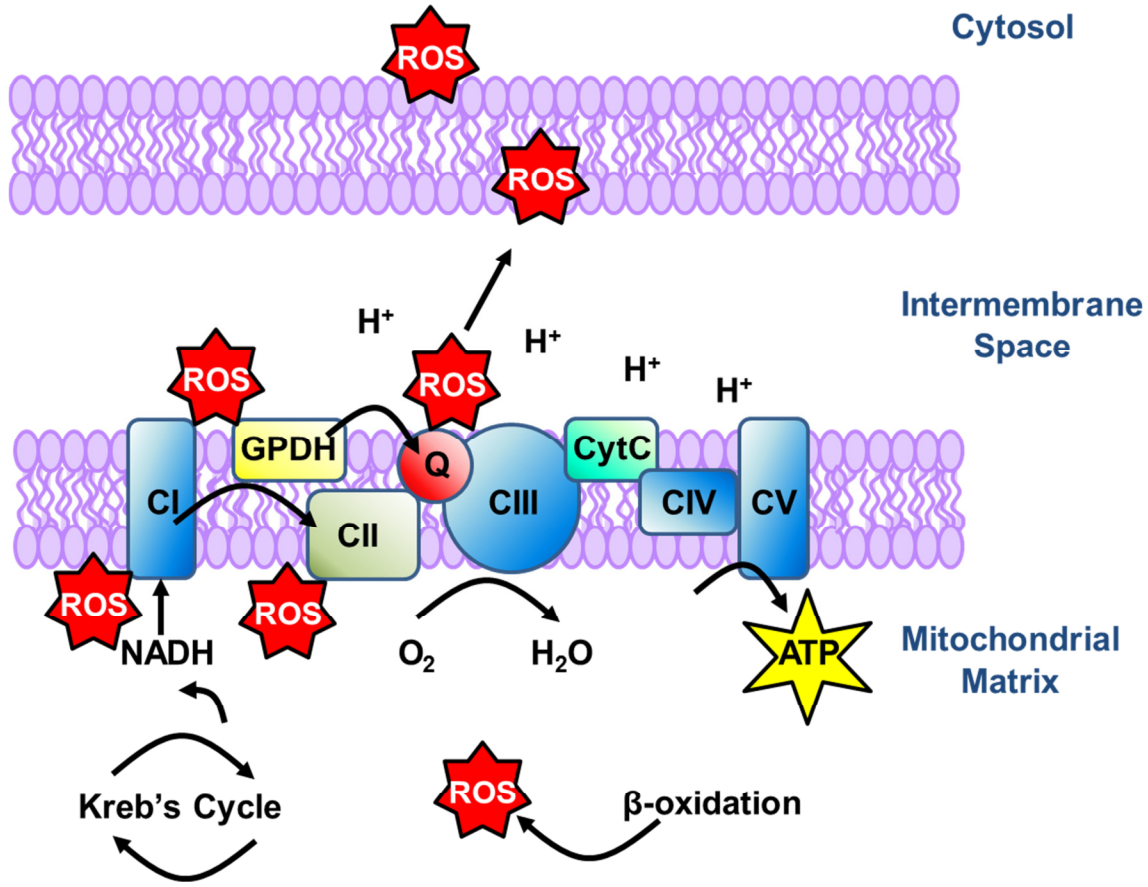


Figure 1.1. Mitochondria and reactive oxygen species (ROS) production.

Complex I-V are denoted CI-CV. 1-3% of the electrons leak before reaching CV, at CI and CIII, reacting with oxygen and forming ROS. The enzyme glycerol – 3-phosphate dehydrogenase (GPDH) also produces superoxide. Adapted from Cloonan and Choi 2013.

Due to their high reactivity, ROS react with DNA, lipids, proteins and carbohydrates, leading to potentially severe oxidative damage (Davies 2000). Many of these processes also trigger chain oxidative reactions leading to much more generalised damage if ROS are not quenched (Halliwell and Gutteridge 2015). For example, lipid peroxidation is a

self-propagating chain reaction, meaning that the initial oxidation of only a few lipid molecules can lead to significant tissue damage (Mylonas and Kouretas 1998).

ROS are, however, not exclusively deleterious; they also play vital roles in cell signalling and gene regulation (Allen and Tresini 2000, Thannickal and Fanburg 2000, Hancock *et al.* 2001, Schieber and Chandel 2014). In particular, ROS are involved in covalent modification of specific amino acids (e.g., cysteine residues; Barford 2004) within redox-sensitive target proteins, which leads to a modification of enzymatic activity (Finkel and Holbrook 2000, Lambeth *et al.* 2007). Many receptor kinases and phosphatases are regulated by ROS, and changes in Ca^{2+} triggered by ROS are responsible for many changes in smooth muscle cell activation (Berridge 2008, Rathore *et al.* 2008).

In addition, ROS (and reactive nitrogen species, RNS) play an important role in immune activation and immune signalling (Bogdan *et al.* 2000, Thannickal and Fanburg 2000, Powers and Jackson 2008). Nitric oxide, a free radical, is one of the key oxidative molecules involved in immunity (Bogdan 2001). It can have either important regulatory functions or serious cytotoxic effects depending on the tissues where this NO is generated and the quantities produced (Nathan 1992). For instance, NO can be produced by constitutive nitric oxide synthases in epithelial cells (nNOS and eNOS), where it is then involved in vasodilation (Wilcox *et al.* 1992) and cell signalling. During immune responses it is produced in large amounts by macrophages and other immune cells through the upregulation of inducible NOS (iNOS) and in this context functions as a killing agent in the fight against pathogens (Rosen *et al.* 1995, Forman and Torres 2002, Minakami and Sumimoto 2006).

To mitigate OS, organisms have evolved highly complex protective systems, termed antioxidants, which include an array of molecules and enzymes (Halliwell 1999, McGraw *et al.* 2010, Halliwell and Gutteridge 2015). Molecular antioxidants can be endogenous, such as vitamin C (Padayatty *et al.* 2003), uric acid (Becker 1993), glutathione (Sedlak *et al.* 2009), melatonin (Tan *et al.* 2002) and bilirubin (Machlin and Bendich 1987), or acquired from exogenous food sources (Kaindl *et al.* 2008, Bouayed and Bohn 2010), including vitamins A (e.g. retinol and carotenes) and E (tocopherols and tocotrienols) as well as thiols (Di Mascio *et al.* 1991). There are also a considerable number of antioxidant enzymes; some of the most abundant in the vertebrate body being hydrogen peroxidase, glutathione peroxidase and superoxide dismutase (Pigeolet *et al.* 1990, Kang and Kang 2013). Antioxidants scavenge free radicals and neutralise them before they cause damage to body tissues (Halliwell and Gutteridge 2015) through donating electrons to unstable compounds that have one or more unpaired electrons (Fig. 1.2). These reactions are catalysed by antioxidant enzymes, thus turning reactive molecules into less or non-reactive ones. The combined ability to quench free radicals is termed antioxidant capacity. Crucially, because of the importance of ROS in signalling, antioxidants have to maintain redox homeostasis; an optimal situation where sufficient functionality of cell signalling and immune protection is ensured, but without causing excessive toxicity (Rhee 2006).

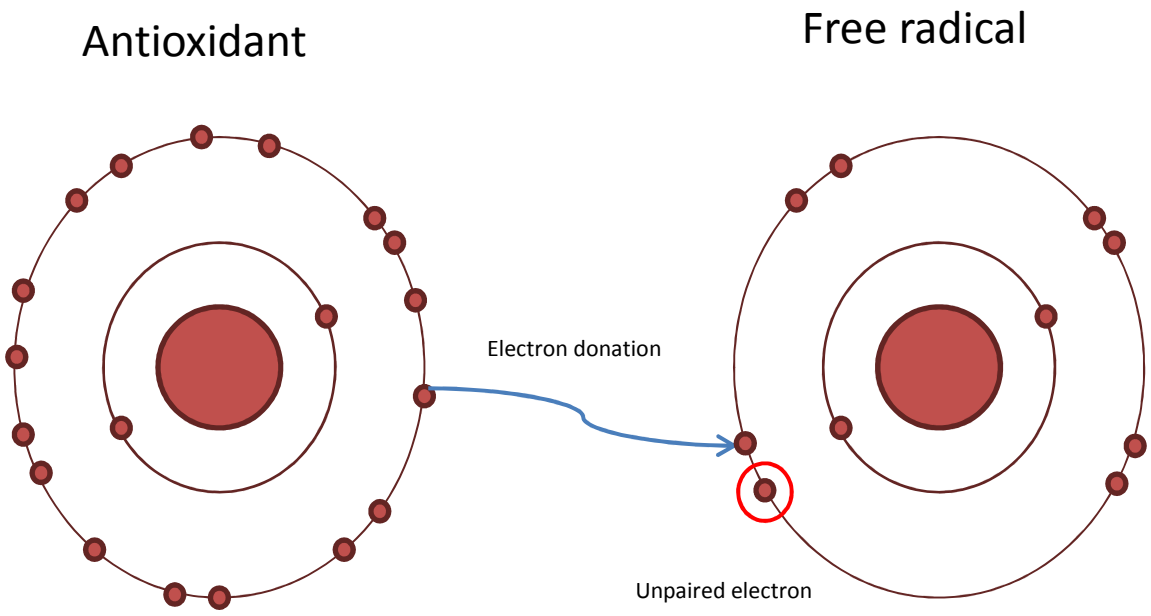


Figure 1.2. Donation of an electron from an antioxidant to a free radical.

Achieving an optimal balance between ROS and antioxidant capacity thus involves trade-offs (Dowling and Simmons 2009, Monaghan *et al.* 2009), where a high metabolic rate – which can be increased by higher energy expenditure for foraging, reproduction and by the need to generate heat through endothermy (Speakman and Król 2010) - leading to greater ROS production, would result in cellular damage. This can potentially result in tumours and organ failures and accelerate the rate of telomere degeneration (Hamilton 1966, Beckman and Ames 1998, Holzenberger *et al.* 2003).

1.1.1 **Ageing**

Senescence, the condition of deterioration with age, can be caused by an increased production of free radicals with age. This is due to an age-related deterioration in capacity to quench free radical action (through antioxidant and / or repair mechanisms) leading to increased oxidative damage (see also Beckman and Ames 1998, Cadenas and Davies 2000), formalised as the free radical theory of ageing (FRTA, Beckman and Ames 1998, Ashok and Ali 1999).

An issue when investigating how senescence influences life-history strategies is the use of cross-sectional studies (Hofer and Sliwinski 2001, Hofer *et al.* 2002, Nussey *et al.* 2008), because individuals that die during a given time-frame might not represent a random subset of a population, requiring that individual age-specific mortality risk be accounted for (Vaupel *et al.* 1979, Vaupel and Yashin 1985, see also Bouwhuis *et al.* 2009, Campbell *et al.* 2012). It is thus necessary to use longitudinal studies in order to differentiate between intra-individual variation and inter-individual heterogeneity (reviewed in Monaghan *et al.* 2008, Nussey *et al.* 2008). In this thesis, I benefitted from 30 years of existing population records and pedigree – enabling me to sample the same, marked individuals over multiple seasons of a 3 year period, creating the longitudinal framework required for such studies.

1.1.2 **OS as a life-history trade-off**

One of the key tenets of life-history theories is that a negative correlation exists between current reproductive investment and future reproductive output (Roff 1992, Stearns 1992, Campbell *et al.* 2012). This is central to the disposable soma hypothesis, where limited resources are allocated either to reproduction, or to other competing factors such as somatic maintenance or immune response (Kirkwood 1977, Klasing 2004). Attaining and / or maintaining reproductive condition, as well as actually producing and rearing offspring (in females), affects OS levels, via increased metabolism and somatic maintenance traded off with reproductive output (birds: Bize *et al.* 2008, reptiles: Isaksson *et al.* 2011b and mammals: Vina *et al.* 2003).

A limitation in oxidative stress ecology has been that most studies consider single physiological processes (OS biomarkers) with respect to isolated fitness or health traits

(Cohen *et al.* 2009, Cote *et al.* 2010, Devevey *et al.* 2010, Isaksson *et al.* 2011b). While this approach has provided valuable insights into life-history constraints, interactions between physiological variables indicate that a wider approach is required (Isaksson *et al.* 2011a).

Oxidative stress is not the only way to assess the physiology of stress in wild animals, with many other techniques having been developed over the years. In particular, corticosteroid measurements measure acute stresses caused by the activation of the Hypothalamic- pituitary-adrenal axis. These measures have been used extensively in conservation to investigate the effects of adverse conditions on fitness and survival (reviewed in Busch and Hayward 2009). Leucocyte coping capacity (LCC), a measure of the oxidative burst ability of circulating white blood cells and neutrophil activation have also been used to measure stress in wild animals (Montes *et al.* 2003). LCC has been showed to vary with acute stressors such as handling and psychological stress (McLaren *et al.* 2003, Gelling *et al.* 2009).

Oxidative stress and the damage it causes have been used to study longer term variations in physiological stress, and the effects of metabolism between seasons and age classes. Indeed, because of its implication in ageing mentioned above, these methods have been showed to have effects measureable over months and years rather than minutes / days measureable with methods such as LCC. Despite the methods being used in both medical and ecological studies for seasonal / long-term studies, short term weather will also be included in analyses to confirm this assumption.

1.2 **Immunity**

Vertebrates are at constant risk of invasion by microorganisms and have evolved immune systems to deal with these pathogens, a risk factor in ecology. In mammals,

innate immunity provides the first line of defence against pathogens, while acquired immunity is involved in the elimination of pathogens as well as development of immune memory (Fearon and Locksley 1996, Akira *et al.* 2001, Hoebe *et al.* 2004).

The innate immune system uses germline encoded pattern recognition receptors (PRRs) to recognise microorganisms. Antigen presenting cells (APCs) act as the link between the innate and acquired immune response, migrating from the site of infection to lymph nodes where they present antigens to naive $CD4^+$ T cells (Fearon and Locksley 1996, Kaufman *et al.* 2015).

One of the key APCs are macrophages (**MΦ**), a type of white blood cell, found in most tissue types and involved in phagocytosis (Aderem and Underhill 1999, Wynn *et al.* 2013); these cells will be the key focus of the immunological section of this thesis. PRRs recognise conserved microbial components termed pathogen-associated molecular patterns (PAMPs; Brubaker *et al.* 2015). These are usually elements essential for the survival of the microorganism, leading to their conservation, but that are not present in the host. These molecules include lipopolysaccharides (LPS) which are endotoxins found in the cell wall of bacteria, flagellin (FGN) a molecule present in the flagella of gram negative bacteria, lipoteichoic acid from gram positive bacteria, peptidoglycans and specific nucleic acid variants such as dsRNA and unmethylated CpG motifs (Akira *et al.* 2006). PRRs are encoded in the germline, and expressed on a variety of cell types. PRRs include a wide variety of receptors including Toll like receptors (TLRs; Takeda *et al.* 2003), RIG-I-like receptors (RLRs; Yoneyama and Fujita 2008), NOD-like receptors (NLRs; Kanneganti *et al.* 2007), and DNA receptors (cytosolic sensors for DNA; Barber 2011) and are known to play a crucial role in host

defence. While RLRs and NLRs sense intracellular molecules, TLRs span the cell membrane to detect extracellular or endosomal molecules (Akira *et al.* 2006).

Damage associated molecular patterns (DAMPs; Gallucci and Matzinger 2001, Benko *et al.* 2008), or danger signals, are endogenous molecules that elicit an inflammatory response within the host. These molecules include cell wall fragments, oxidative stress molecules and heat-shock proteins. For example, the presence of DNA outside of the mitochondria or nucleus is picked up by Toll-like receptor 9 (TLR9) and starts an inflammatory cascade (Zhang *et al.* 2010). TLRs also recognise heat shock proteins and misfolded proteins, or exposed hydrophobic parts of molecules (Lotze *et al.* 2007). Mitochondrial DAMPs (mDAMPs) signal to the cell that the mitochondrion is damaged – as can arise from high levels of oxidative stress, autoimmunity, micronutrient deficiency or disease (Jain *et al.* 1991, Bolaños *et al.* 1996, Kowaltowski and Vercesi 1999). Mitochondria contain several unique structures such as cytochrome c, mitochondrial DNA and formyl peptides that can signal damage (Shimada *et al.* 2012). For example, cytochrome c, when released from the mitochondrion inner membrane to the cytosol, acts as an apoptotic signal (Kluck *et al.* 1997).

The outcomes of **MΦ** activation by PRR induce production of immune mediators such as nitric oxide and cytokines to fight pathogens and direct the activities of other cells (Kawai and Akira 2008). Oxidative stress can also be the cause of upregulation of pro-inflammatory cytokines (Macdonald *et al.* 2003), which in turn can cause oxidative stress (Lavieri *et al.* 2014). This type of process can lead to chronic inflammation. Because of its high reactivity, nitric oxide can also lead to severe damage to the host, which is why its production by the immune system is highly regulated.

Possibly the best defined PRRs are the family of TLRs (Kawai and Akira 2010). Toll is a gene originally identified in Drosophila (*Drosophila melanogaster)* as being essential for dorso-ventral axis formation, but was also found to be involved in anti-fungal responses (Lemaitre *et al.* 1996). TLRs, while having a similar overall structure, only have immuno-regulatory roles (Takeda and Akira 2005). They have been found in primitive species such as *Caenorhabditis elegans* (a free-living nematode; Tenor and Aballay 2008), and are conserved across the vertebrates, with the six mammalian TLR families conserved from pufferfish to humans (Roach *et al.* 2005). And while plants do not have TLRs, they have functionally equivalent PRRs that recognise PAMPs (Zipfel 2009). This high level of conservation of TLRs suggests that there is a strong selective pressure to recognise PAMPs. The principal TLR families can be grouped according to the PAMPs they recognise (Fig. 1.3). For example, TLRs 1, 2 and 6 recognise lipids while TLRs 3, 7, 8 and 9 recognise nucleic acids (Schwandner *et al.* 1999, Hemmi *et al.* 2000, Liu *et al.* 2008, Mancuso *et al.* 2009).

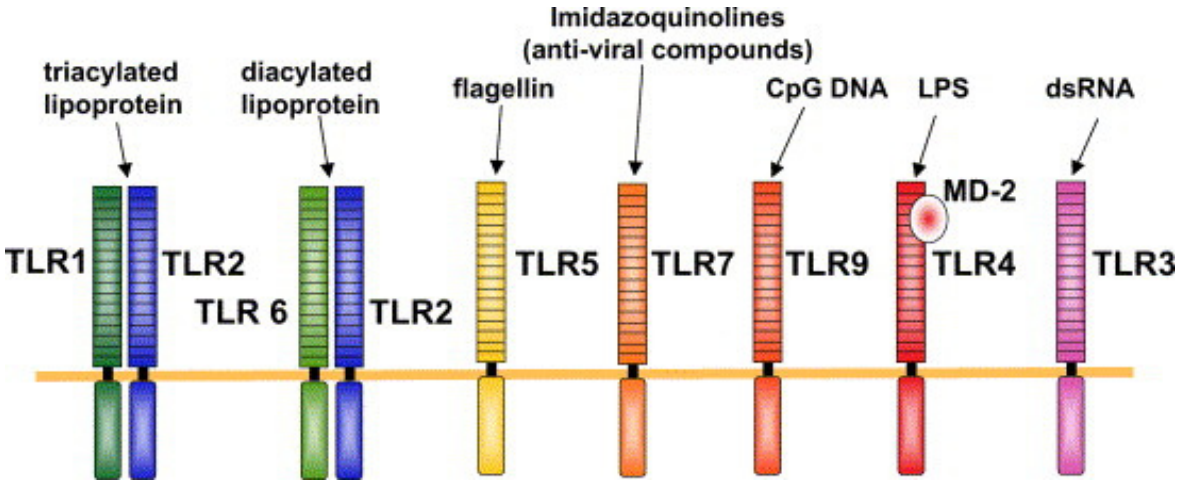


Figure 1.3 TLR families and the PAMPs they recognise. From Takeda and Akira 2004.

Many cells that express TLRs are APCs, including the previously described MΦ, as well as being expressed on B cells, specific T cells and on non-immune cells (fibroblasts and epithelial cells). Some TLRs are found on the cell surface (1, 2, 4, 5 & 6), while others (3, 7, 8 & 9) are found in the intracellular endosomal compartments (Takeda and Akira 2005). Expression of TLRs is rapidly modulated in response to pathogens, cytokines and cellular environmental factors (Moynagh 2005, Kawai and Kawasaki 2015).

1.2.1 Outcomes of TLR signalling

1.2.1.1 Cytokine production

Cytokines are a small group of proteins involved in cell signalling, where each cytokine can typically be produced by a number of different cell types (Murphy and Weaver 2016). Cytokines include molecules such as interleukins (ILs), tumour necrosis factor (TNF), Interferons (IFNs), lymphokines and chemokines (Murphy and Weaver 2016, see also Lata and Raghava 2008). They are important immune system proteins, involved in the host's response to pathogens, and upregulation of these genes can be measured as indicators of immune activation (Hayashi *et al.* 2003). There is a matching cell surface receptor for each cytokine, and - when bound - they cause a downstream cascade of intra-cellular signalling that will alter cell function such as causing a change in the regulation of genes and transcription factors (Benton 1991).

The diversity of cytokines (see Table 1.1 for examples of principal cytokines), is linked to their wide variety of functions and targets. In the immune system, pro-inflammatory cytokines control infection by up-regulating inflammatory responses (Alcendor *et al.*

2012, Duque and Descoteaux 2015), whereas anti-inflammatory cytokines down-regulate inflammatory responses (Opal and DePalo 2000).

Cytokines have had many different classifications over the years, but the most useful for our purpose divides immunological molecules into two categories, molecules that enhance cellular immune responses (e.g. TNFα, IFNγ, IL1β; Bartholdi and Schwab 1997) and those that facilitate antibody responses (e.g. IL4, IL10; Fiorentino *et al.* 1991, Hochrein *et al.* 2000).

Part of this dual strategy is to make sure that the immune system does not over-react to pathogens and cause excessive damage to host tissues. By having positive and negative feedback loops, organisms can regulate an immune reaction by first initiating a strong pro-inflammatory reaction, which is later reduced in favour of a more specific response by the acquired immune system (Renner and Schmitz 2009). These anti-inflammatory circuits can, however, be hijacked by pathogens where the pathogen manipulates the host's anti-inflammatory circuits to avoid being killed off by the immune system (e.g. TB; Gupta *et al.* 2012). The malfunction of this regulatory mechanism has also been investigated with regards to pathogenesis and auto-immune disorders (Poletaev *et al.* 2012, Rönnblom and Eloranta 2013).

Table 1.1. Principal cytokines, their sources and activity

Cytokine	Principal source	Principal activity
IL1β	Macrophages	T and B cell activation, inflammation
IL6	Macrophages and T cells	B cell stimulation, inflammation
IL10	Th2 cells and macrophages	Inhibits Th1 cytokines, enhances B-cell production
IL12	Antigen presenting cells	Stimulates T cells into Th1 cells, stimulates IFNγ
IFNγ	Th1 cells, natural killer cells, macrophages	Proliferation and differentiation of macrophages
TNFα	Macrophages, Th1 cells	Inflammation, tumour killing, neutrophil activation

1.2.1.2 **Nitric oxide (NO)**

Nitric oxide is a free radical which, is a signalling molecule involved in many physiological and pathological functions. NO is a powerful vasodilator, crucial for the effective functioning of the cardiovascular system (Lei *et al.* 2013), and it is also one of the key antimicrobial molecules produced by phagocytes as part of an immune reaction, where its high toxicity and reactivity damage invading pathogens (Bogdan 2001). NO is of key importance in the control of tuberculosis (Mishra *et al.* 2013). As TB is one of the most economically important wildlife diseases, particularly in badgers (Woodroffe and Donnelly In press), the upregulation of NO in badgers will be investigated in Chapter 7 of this thesis.

There are three genes responsible for producing NO, NOS1, NOS2 and NOS3. Neuronal NOS (nNOS or NOS1) and epithelial NOS (eNOS or NOS3) are also known as cNOS because they are constitutively expressed and activity is Ca^{2+} dependent (Agulló *et al.* 1995). These two isoforms are now recognised to be widely distributed in tissues and affect cell differentiation and cell signalling *in vivo* and *in vitro*. Inducible nitric oxide synthase (iNOS or NOS2), is a homodimeric, heme containing enzyme which converts L-arginine and oxygen to L-citrulline and NO by oxidoreduction

(Clancy and Abramson 1995). iNOS is characterised by its lack of expression in resting cells, but is inducible in a Ca^{2+} independent manner (Aktan 2004). The main product released from the heme complex within NOS2 is the NO radical (NO˙). It reacts with many partners to include both toxic and regulatory effects. Most mammalian species upregulate iNOS in response to TLR stimulation as an antimicrobial mechanism, but stimulation by IFNγ is a common alternative route for inducing NO production (Fig. 1.4, Aktan 2004).

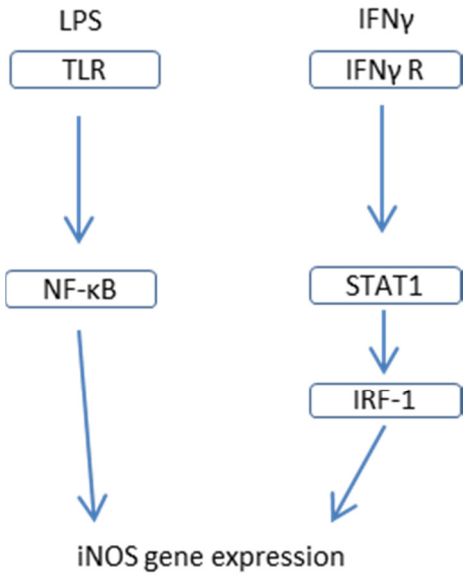


Figure 1.4 Regulation of iNOS gene expression through TLR or IFNγ stimulation.

NF-κB is a DNA transcription factor, STAT1= Signal transducer and activator of transcription 1, IRF1= Interferon regulatory factor 1. Simplified from Aktan 2004

The investigation of immunology in wild mammals has only recently become a major interest to ecologists worldwide. Immuno-ecology investigates the immune system in relation to the environment, ecological setting and potential trade-offs required to maximise fitness (Sheldon and Verhulst 1996, Martin Ii *et al.* 2006, Demas and Nelson 2012). It attempts to give an evolutionary perspective on specific immune mechanisms and explain natural variation in immune responses (Viney and Riley 2014). Using the

badger as a model, we investigate the links between the ecology, physiology and immunology, and the impacts these factors could have on survival and reproduction.

The study of immuno-ecology is often limited to simple but non-specific methods which are easy to achieve in field conditions, or that can be accomplished by non-specialist personnel. These include techniques such as parasite counts or subcutaneous stimulation with immuno-stimulants (Phytohaemagglutinin (PHA) or LPS; Kennedy and Nager 2006). The latter requires subcutaneous injection of PHA or LPS and measuring the amount of reaction (swelling) in response to the stimulation when compared to a control of physiological solution (Costantini 2008). The advent of easily accessible molecular techniques has changed the face of immuno-ecology and allowed for a more in depth study of the immune system of wild animals. This aspect of the field is still in its infancy, however and most early molecular based immuno-ecology has been done on rodents where sufficient genomic resources exist for advanced molecular work on wild populations (Pedersen and Babayan 2011). There is another option whereby researchers can use the genome of a closely related species in order to develop new assays (see Chapter 6).

Diverse and repeated exposures to infection are common for wild animals, mitigated by two strategies (Schneider and Ayres 2008): a) Resistance, where individuals mount a significant immune response in order to clear the infections (Mackaness 1962), but this could require substantial metabolic resources, as well as the risk of incurring oxidative damage. b) Tolerance to infection, where individuals limit the infection's growth, without expending excessive resources, or incurring substantial oxidative damage; however, this risks infection getting out of control, especially during periods of

physiological stress or immune depression such as during pregnancy (Restif and Koella 2004, Paun and Danska 2015). The potential for a trade-off exists where different infections and pathogens in the environment require a different level of immune responses. If the infection is likely to be repeated, there are several options: either individuals fight it off without too much damage and become immune, or if they cannot fight it off easily or are likely to incur too much physiological damage from mounting a big immune response, in which case it is probably better to contain the infection (Hirano *et al.* 1983).

A current perspective is that a tolerant response could have counter-productive effects at the population level, as it could lead to the presence of super-spreaders (Paull *et al.* 2012): i.e., individuals who carry an infection but tolerate it rather than clear it, while remaining contagious. These individuals can secure their own fitness while putting the rest of the population at risk. This is of particular importance for endangered populations that are in close enough proximity for super-spreaders to migrate between groups (Stein 2011). Individuals mounting a strong immune response may be more likely to damage their tissues. Conversely, an individual might not respond strongly to a certain pathogen, making it more susceptible to the disease. This does not, however, mean that its lifetime reproductive output will necessarily be affected (Johnson *et al.* 2012). On the contrary it might be that this individual's immune strategy tolerates limited damage, but simultaneously limits self-damage because of its low level of immune response.

Another field of biology where ecology, immunology and physiology interlink are zoonotic epidemics (Karesh *et al.* 2012). Badgers are involved in the maintenance of

bovine TB in the UK, acting as a wildlife reservoir (Macdonald *et al.* 2006, Brooks-Pollock *et al.* 2014, Donnelly and Woodroffe 2015, Macdonald *et al.* 2015b). The failure of current eradication policies and the possible high susceptibility of badgers throw up many questions surrounding the immune responses of badgers to pathogens, and TB in particular.

1.2.2 **Tuberculosis**

Tuberculosis is caused by certain species of the *Mycobacterium* genus; a group of actinobacteria also containing the bacteria responsible for leprosy (Admassu *et al.* 2015.) Mycobacteria are aerobic bacteria characterised by their particularly thick, waxy, hydrophobic cell wall which is rich in mycolic acid (Fig. 1.5, Niederweis *et al.* 2010). The structure of their cell wall makes these infections particularly difficult to treat, and mycobacteria are naturally resistant to many antibiotics that disrupt cell wall synthesis (Jarlier and Nikaido 1994). These features are also thought to be important in clinical latency as many species, including those responsible for tuberculosis can colonise hosts without them being symptomatic (Flynn and Chan 2001b, Gupta *et al.* 2012). This means that individuals can be infectious without any obvious symptoms, making it difficult to target individuals in an ecological setting, where extensive testing for the disease might not be feasible.

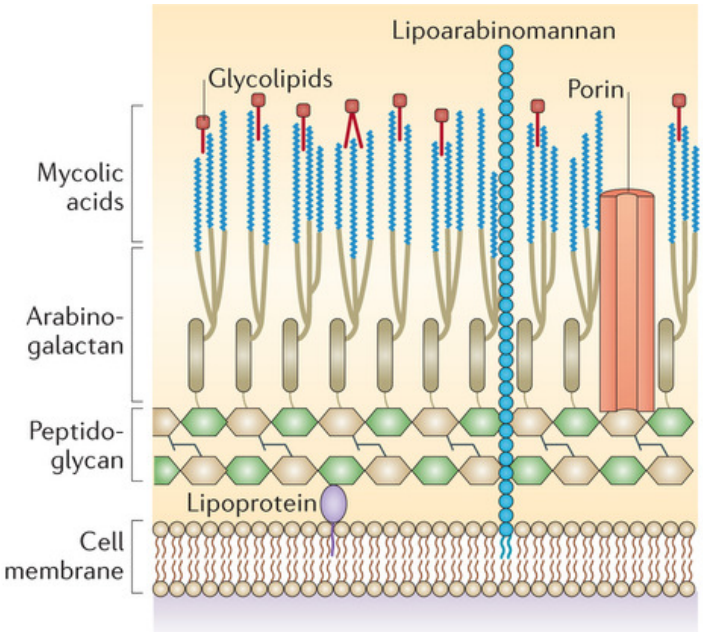


Figure 1.5 Structure of the mycobacterial cell wall. From Brown *et al.* 2015

1.2.2.1 TB in biomedical species - typical pathogenesis

Many studies have investigated the pathogenesis of TB infections and the immune responses of species of biomedical importance (e.g. mice and humans; Smith 2003, Orme 2014). In human hosts, uncontrolled proliferation of TB bacteria generally causes severe lung lesions and damage that eventually leads to suffocation. This occurs as the bronchiolar passages become obstructed by granulomas and fluids, and the parenchymal cells required for oxygen uptake are destroyed by the disease. Inflammation is a key issue in TB pathogenesis, as the immune responses required to control the disease contribute to the considerable tissue damage occurring in the lungs (Rook and Hernandez-Pando 1996). iNOS, and cytokines such as TNFα, IL12p40 and IFNγ are key in the control of TB infections, and are especially required for the formation of granulomas that reduce the dissemination of the bacteria (Senaldi *et al.* 1996, Reece *et al.* 2010). Despite the importance of TNFα in controlling the disease, excess production of this cytokine leads to chronic inflammation and early death in mouse models (Bekker *et al.* 2000).

1.2.2.2 **TB in livestock**

Tuberculosis is an important economic and health issue in livestock, costing the UK tax payer £100million / year (DEFRA 2011). Many European countries are considered to be TB free, with only few minor outbreaks each year (see Humblet *et al.* 2009). Bovine TB was once almost eradicated from Great Britain, but since the mid-1980s the disease has spread and increased exponentially in prevalence (Gilbert *et al.* 2005). This increase and the toll on cattle farming have led to considerable political debate on the measures to control the spread of bTB in cattle (Woodroffe and Donnelly In press).

There are many potential wildlife reservoirs for bovine tuberculosis throughout the world. In the UK, badgers are considered the most important wildlife reservoir and the relative importance of badgers (versus cattle) has stimulated intense political and scientific debate over the last 20 years (Woodroffe and Donnelly In press). In order to control the spread of bTB, the Randomised Culling Trial aimed to investigate the possibilities of using culling as a mechanism for controlling the badgers to reduce the risk of TB transmission from badgers to cattle. The cull trial was specifically targeted on areas with high endemic bTB and featured three cohorts; no culling, generalised culling or culling in response to outbreaks in local cattle. The authors of the report claimed that whilst generalised culling did decrease the number of outbreaks in the immediate area, it increased the range size of the remaining badgers and actually increased the number of outbreaks in areas around the culling circle (Godfray *et al.* 2004, Donnelly *et al.* 2006a, Woodroffe and Donnelly In press). This has left the situation currently unresolved, with suggestions for culling to be combined with vaccination (Chambers *et al.* 2014).

1.2.2.3 **Vaccination of wildlife**

Vaccinating wildlife has been used to protect public and livestock health, or as a means to protect endangered species. Vaccination is the most efficient in species where host birth-rate and death-rate are low and disease propagation is low (Keeling *et al.* 2003).

Bacillus Calmette–Guérin (BCG), the only commercially licenced vaccine against TB, has a protective effect in captive and wild badgers via intradermal, subcutaneous, mucosal and oral vaccination (Corner *et al.* 2008, Carter *et al.* 2012b, Robinson *et al.* 2012). Vaccinated badgers exhibit less severe infection, characterised by fewer gross lesions, fewer sites with histological lesions and lower bacterial load in the lungs (Chambers *et al.* 2014). Cell mediated immunological responses to BCG are lower or absent in comparison to responses in badgers infected with virulent *M. bovis* (Corner *et al.* 2010). Critically, badger cubs from the same social group are also 70% less likely to test positive to TB, demonstrating reduced transmission (Carter *et al.* 2012a). Combining vaccination with immunological measurements could provide crucial information on how best to vaccinate wild animals and what potential changes need to be made to the original vaccine in order to tailor it more specifically to the focal species.

Chapter 2: **General methods**

2.1 **The European badger as a wild mammal model**

The European badger (*Meles meles*) is an omnivorous, medium sized mustelid, widespread across Europe (Johnson *et al.* 2002b) with the closely related species *M. leucurus* in Asia and *M. anakuma* in Japan (Wilson and Reeder 2005). The badger provides an ideal model species for ecological and eco-physiological research because it is abundant in the UK study region, easy to trap and sufficiently large to allow necessary blood volumes (<10 % blood volume at 40 ml/kg) to be collected without this being of animal welfare concern, even in cubs (see Macdonald and Newman 2002, Montes *et al.* 2003, 2004).

A study of badger socio-ecology has been on-going since 1987 (Macdonald et al. 2015) in Wytham Woods (Fig. 2.1) - a 424 ha site of Special Scientific Interest (Savill et al. 2012), comprised of semi-natural mixed woodland, situated 5 km North West of Oxford, (01° 18’ W, 51° 46’ N).

2.2 **Trapping Protocol**

Routine badger trapping provides detailed, long-term demographic data for this geographically discrete population. Importantly, this study has progressively refined handling protocols over the past 25 years in order to minimise stress, which could otherwise bias physiological measurements (Montes *et al.* 2003, 2004, Thornton *et al.* 2005; Sin et al. 2015). Trapping took place 3 times / year (seasonally: spring, summer, autumn) using steel mesh traps baited with peanuts following the methods described in Macdonald & Newman (2002). Trapping was suspended from January to May during the final two trimesters of gestation and until suckling juveniles were fully weaned.

Traps were deployed on badger paths radiating from sett entrances at distances of 1-5 m, and baited with peanuts late afternoon. Each active sett was trapped for 3 successive nights. Note: No pre-baiting was used in this study, and thus the amount of food supplied to the population through trapping is energetically inconsequential for population dynamics (Macdonald *et al.* 2009)

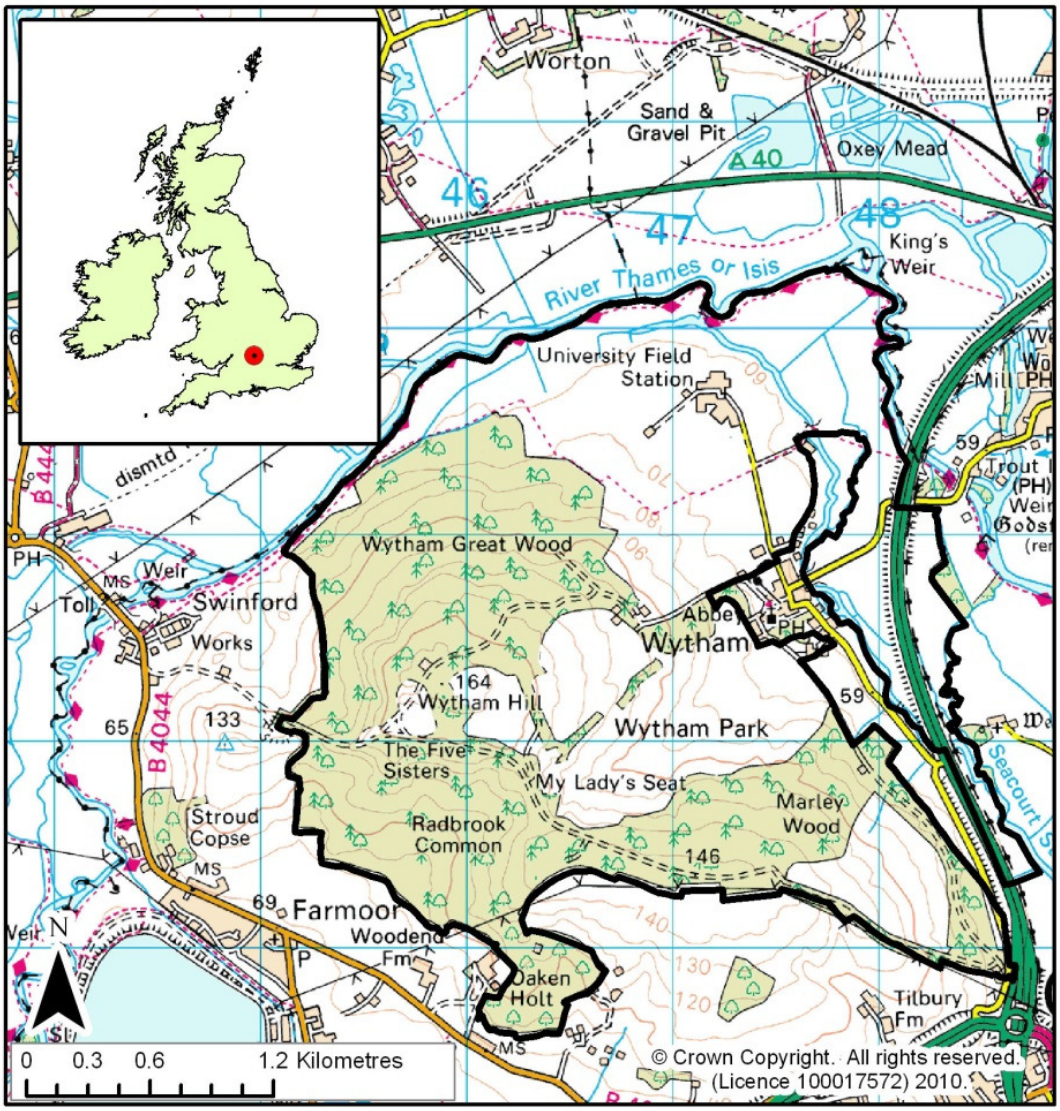


Figure 2.1. Map of Wytham Woods, Oxfordshire, UK

Badgers were collected between 6.30 and 8 am, transferred to smaller holding cages and transported to a central handling facility in a cargo vehicle or a trailer towed by an all-terrain vehicle (Montes *et al.* 2011). Individuals were sedated with an intramuscular

injection of ketamine hydrochloride at 20 mg / kg (Mackintosh *et al.* 1976, McLaren *et al.* 2005, Thornton *et al.* 2005) and, upon first capture, given a permanent identifying tattoo in the left inguinal region (Cheeseman and Harris 1982). While under sedation, morphometric data were collected including weight, body-condition (subcutaneous fat score of 1-5; Speedy and Clark 1981), reproductive condition, ectoparasite counts, as well as multiple samples for genetic and physiological analysis.

Because of the effects of transportation on stress in wild badgers (Esteruelas *et al.* 2016, Montes *et al,* 2003), this must be considered when interpreting overall results of assays conducted on animals which have been transported and handled. In order to minimise the effects of these stressors on sampling, badgers were rested following transport.

All trapping and sampling were carried out under Natural England licence (20123461 and 2013331) and Home Office licence PPL 30/2835.

2.3 **Blood sampling**

Blood samples were collected from spring 2012 until August 2014, between 8.30 and 11 am, to minimise circadian variations. Individuals were marked with a temporary blue dye livestock marker to avoid subsequent re-sampling during the same trapping sessions – unless required by specific protocols. For example, for the purpose of OS method validation (see Chapter 3), 20 individuals were deliberately re-sampled on a non-consecutive day within the 2-week trapping.

Approximately 12 ml of blood (never more than 5% estimated badger blood volume by weight) were collected from the jugular vein in heparinised vacutainers (BD Vacutainer® systems, Plymouth, UK) using 21'G x 1½" needles for immunological and oxidative stress assays.

Blood was prepared for physiological and immunological assays in 4 steps:

1) 20 µl of full blood was diluted in 730 µl of 'KRL mammal assay buffer' (Kirial, Courernon, France) and stored in micro-centrifuge tubes at 4ºC until analysis, within 12 h of blood collection.
2) The remaining full blood was then centrifuged for 10 min at 1500 g (4ºC). The plasma was aliquoted and frozen at -20ºC immediately on site, before being transferred to -80ºC at the end of each week of trapping.
3) Once the plasma was removed, 10 µl of red blood cells (obtained by centrifugation) were diluted in 740 µl of 'KRL mammal assay buffer' and stored under the same conditions as (1).
4) As of June 2013, the remaining blood components were re-suspended in phenol red-free Dulbeco's Modified Eagle Medium (31053-044, Gibco, Life Technologies Ltd, Paisley, UK) containing 10 % foetal calf serum (FCS) (Gibco, Life Technologies Ltd, Paisley, UK), 2 mM Penicillin and Streptomycin (Gibco, Life Technologies Ltd, Paisley, UK), and 2 mM L-Glutamine (G7513, Sigma-Aldrich, Gillingham, UK) and kept at 4°C. White blood cell extraction was performed within 8 h of blood collection (see below).

2.4 **Oxidative stress measurements**

Oxidative stress is caused by an imbalance between pro- and anti-oxidants (see Chapter 1), leading to oxidative damage to biological structures such as lipids membranes, protein and DNA (Cadenas 2000). For this reason it is important to consider measurements in combination with other elements of the balance. For example, measuring high levels of antioxidants does not necessarily mean that an individual is doing well, instead, it could have needed to upregulate it's antioxidant defences because

of high levels of ROS, which could lead to oxidative damage (Halliwell and Gutteridge 2015, & Monoghan, Metcalf and Torres 2009). This is why in this study; I tried to measure a variety of OS markers (circulating antioxidants, enzymatic antioxidants, resistance of cell walls to oxidative stress as well as oxidative damage.

For all assays, samples were run in duplicates with the appropriate negative and positive controls and measurements performed in a 96 well plate spectrophotometer (FLUOstar OMEGA 415-0435, BMG LABTECH GmbH, Germany). For all absorbance assays, Grainer flat bottomed 96 well plates were used (Greiner Bio-One Ltd., UK).

2.4.1 **Red blood cell killing assay (½-life)**

The red blood cell (RBC) killing assay, uses the capacity of RBCs to resist lysis in the presence of a strong oxidant as a proxy for oxidative stress resistance, measured as RBC ½-life. 135 µl of 150 mM AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride, Sigma-Aldrich, Dorset, UK) were added to 90 µl of diluted blood (full blood, or RBC, as described under the blood sampling protocol. Assays were started within 48 h of blood sample collection. Absorbance was measured spectrophotometerically at 450 nm, every 2.5 min for 3 h. The plate was maintained at 37ºC for the entirety of the reaction, and the machine was programmed to shake the plate before every measurement to avoid sedimentation of the RBCs. RBC ½-life was calculated using the following method: Absorbance values were plotted against time, and these data were smoothed using a quadratic curve (Fox and Weisberg 2010). Half-life was calculated as the time for the initial absorbance to halve. Mean values were calculated from sample duplicates.

2.4.2 **Uric acid**

Uric acid is a waste product of purine metabolism with antioxidant properties that play an important role in quenching ROS (Smith and Lawing 1983). Uric acid was measured using a commercial kit (KA1651, Abnova Corporation, Taiwan), where 2,4,6-tripyridyl-s-triazine forms a blue complex with iron in the presence of uric acid, measurable spectrophotometrically at 590 nm. Following the manufacturer's instructions, 5 µl of plasma were mixed with 200 µl of working reagent (10 volumes reagent A, 1 volume reagent B, 1 volume reagent C). The plate was incubated at room temperature for 30 min before reading the absorbance at 590 nm. Values were then calculated by comparison to a uric acid standard.

2.4.3 **Total antioxidant capacity (AOX)**

A multitude of biochemical assays exist to measure antioxidant capacity based on antioxidant enzyme activity, non-enzymatic antioxidant capacity and repair mechanisms. Following Costantini et al. (2007) and Isaksson et al. (2011), antioxidant capacity was measured as total, non-enzymatic, circulating antioxidants (AOX). This type of assay measures the potential of endogenous and dietary molecules present in the plasma to protect against free radical activity *in vitro*. AOX was measured using a commercial kit (STA-360, Cell Biolabs, San Diego, USA) where antioxidants reduce Cu^{2+} to Cu+ (Özyürek, Kubilay, and Apak 2011), which reacts with neocuproine to form an orange chromogen measurable at 490 nm. There are many different assays used to measure non-enzymatic antioxidant capacity, most of them based on the redox status of metals such as copper and iron. Colorimetric assays like the one used here tend to be slightly less specific and sensitive than fluorometric assays, with fluorescent probe assays being the most specific (reviewed in Cao and Prior 1998).

20 µl of plasma was added to 180 µl of reaction buffer in a 96 well plate. Blank absorbance was measured at 490 nm and 50 µl of copper ion reagent was added. The plate was incubated on an orbital shaker for 5 min, before the addition of 50 µl of stop solution. Final absorbance was read at 490 nm. To calculate AOX as uric acid equivalent units, blank values were subtracted from the final reading and values were compared to a uric acid standard curve.

2.4.4 **Hydrogen peroxide / Peroxidase**

Hydrogen peroxide (H_2O_2) is a key ROS involved in OS. Peroxidase is one of the enzymatic antioxidant mechanisms responsible for quenching hydrogen peroxide. Hydrogen peroxide and peroxidase were measured using a fluorometric assay kit (STA-344, Cell Biolabs, San Diego). In this assay, hydrogen peroxide reacts with ADHP in the presence of horseradish peroxidase (HRP) to produce resorufin, which can be measured fluorometrically. Fluorometric assays are more accurate and sensitive than colorimetric assays (Zaitsu and Ohkura 1980), however there are now even more sophisticated methods for measuring H2O2 which include fluorescent probes which also allow the quantification of transient changes in H2O2 within a cell (reviewed in Rhee *et al.* 2010).

50 µl of plasma was added to 50 µl of reaction mix containing 100 µM of ADHP and HRP (0.2 U/ml, for H_2O_2 assay) or H_2O_2 (2 mM; for peroxidase assay) in the wells of a black 96 well plate (Nunc, Sigma-Aldrich, Dorset, UK). The plate was then incubated in the dark for 30 min before reading the fluorescence (excitation 530 nm, emission 590 nm). Hydrogen peroxide / peroxidase content was then calculated by comparison to a standard curve.

2.4.5 **Malonaldehyde (MDA)**

When antioxidant protection or repair is not sufficient, oxidative damage accumulates, which can have an impact on survival. Oxidative damage can be assessed as protein, lipid or DNA oxidation. When working with mammal blood (non-nucleated RBC), DNA oxidation is not practical to measure and would require large amounts of blood to access white blood cell DNA. Here, I measured MDA (lipid peroxidation product) levels using a commercial thiobarbituic acid reactive species (TBARS) assay kit (STA-330, Cell Biolabs, San Diego, USA). Two molecules of thiobarbituric acid react with one molecule of MDA to produce a pink molecule with a peak absorbance at 532 nm. TBARS has been criticised for being non-specific to MDA. Indeed, the assay is known to interact with other physiological componenents such as bilirubin (Knight , Pieper and Mc Clellan 1988, Janero 1990) and some fatty acids (Pryor and Blair 1976, Tarladgis and Watts 1960). Additionally, the protocol can sometimes lead to chain lipid peroxidation reactions within the sample, thus biasing the results. A full review of the advantages and disadvantages of measuring MDA is presented by Grotto et al. (2009). Despite this, TBARS are still used extensively in ecological and biomedical research (REFs) and provides a cheap and fast method to measure lipid peroxidation. In order to avoid further peroxidation of the sample during the assay, butylated hydroxytoluene (BHT) was added to samples in a final concentration of 0.05 % (Pikul *et al.* 1983). Following the manufacturer's protocol for hydrophilic samples, 100 µl of sample, or standard, was incubated with 100 µl of sodium dodecyl sulphate (SDS) lysis solution for 5 minutes. 250 µl of TBA reagent (pH adjusted to 3.5) was then added and incubated at 95ºC for 60 min. Samples were centrifuged for 15 min at 3000 g, and the supernatant (300 µl) was re-suspended in 300 µl of N-butanol. This was vortexed for 2 min, followed by centrifugation at 30,000 g for 5 min. The butanol fractions were

transferred to a 96 well plate and absorbance was measured at 532 nm. Concentrations of MDA were then calculated by comparisons to a standard curve.

2.5 **Immunology**

2.5.1 **Cell culture**

In order to further investigate the molecular aspects of physiology and immunology, we used cell culture methods to investigate badger macrophages. Peripheral blood monocytes were isolated by overlaying blood re-suspended in medium onto an equal volume of Ficoll-Paque PREMIUM (GE Healthcare, Life Sciences, Buckinghamshire, UK) for centrifugation (1200 g, 30 min, 18°C, break switched off). Cells at the gradient interface were collected and re-suspended in 50 ml of ice-cold PBS with 2% FCS. Cells were washed 3 times and pelleted by centrifugation (1200g, 5 min, 4°C) before being re-suspended in 5 ml cell culture medium (composition as described above).

Cells were counted using a haemocytometer, using trypan blue exclusion to assess viability. Cell numbers were adjusted to $5x10^6$ cells / ml. 100 µl of this solution was then put into each well of a 96 well plate and incubated for 48 h at 37°C, 5 % CO_2 to allow for differentiation into macrophages. Cells were washed after 24 h to discard non-adherent white blood cells and treated with various stimuli as described in subsequent sections and chapters. After the treatment period, cells were stored in RLT buffer (Qiagen, Manchester, UK) and stored at -80°C until used.

2.5.2 **Freezing of badger cells**

For long term storage, surplus blood cells were frozen after ficoll isolation at a concentration of 10^7 cells / ml in liquid nitrogen. To cryo-protect the cells, they were resuspended in DMEM with 10% DMSO and 20% FCS (final volume). Cryogenic vials

were placed in isopropanol freezing containers at -80°C for slow freezing overnight before being transferred to liquid nitrogen.

When cells were required, vials were removed from liquid nitrogen and placed in a 37˚C water-bath before cells were re-suspended in ice cold cell culture medium. Cell were washed 3 times to remove traces of DMSO before being counted as describe above.

2.5.3 **Nitric oxide**

Quantification of nitric oxide produced by macrophages was performed using a commercial Greiss reaction kit (G2930, Promega, Southampton, UK) where absorbance was determined spectrophotometrically at 520 nm (FLUOstar OMEGA 415-0435, BMG LABTECH GmbH, Germany) and concentrations calculated from a nitrite (NO_3^-) standard curve.

2.5.4 **Gene expression**

To quantify changes in gene expression, messenger RNA (mRNA) was extracted from treated and non-treated cells, transformed into cDNA, and analysed by qRT-PCR. The number of mRNA copies in each sample was calculated by comparing to a relative, or absolute, standard curve.

2.5.5 **RNA extraction**

RNA was extracted from cells frozen in RLT buffer using RNeasy mini kit (Qiagen, Manchester, UK) or RNeasy 96 well plates with DNase digestion following the manufacturers protocol. RNeasy kits use a silica based membrane to bind RNA molecules longer than 200 base pairs (bp). RNA concentration was measured by nano-

drop to standardised quantities used for cDNA synthesis and frozen at -80°C until needed.

2.5.6 **cDNA synthesis**

cDNA was synthesised using First Strand cDNA kit (E6300, New England Biolabs, Hitchin, UK) or High capacity cDNA reverse transcriptase kit (Applied Biosystems, UK) following the manufacturer's instructions and frozen at -80°C until used. Both cDNA kits were tested for comparison, and yielded similar quantities and quality of cDNA from the same RNA.

2.5.7 **qRT-PCR**

Intron spanning qRT-PCR assays were designed using badger gene sequences (see Chapter 6). Assays were tested for specificity by melt curve analysis (StepOne software), and cloning and sequencing of amplicons. Validated assays were then used to analyse cytokine cDNA levels in treated macrophages (see Chapters 6 & 7). 2 µl of primer, 4 µl of H_2O and 4 µl of cDNA was added to 10 µl of FAST SYBR green mastermix (Thermo Scientific, UK).

Dilution series of linearised plasmids containing cytokine or TLR gene sequences were used to calculate copy numbers (for development and linearisation of plasmids see Chapter 6).

2.5.8 **Cloning**

TA cloning allows target sequencing, and was therefore performed to confirm nucleotides at potential single nucleotide polymorphisms (SNPs) apparent in direct sequencing. Sequencing primers for TA cloned products are also more robust than general PCR primers, making the sequencing process more efficient.

RT-PCR products were prepared for cloning using Minelute cleanup kits, according to manufacturer's instructions. These products were then cloned using pGEM®-T Easy Vector Systems for small fragments (<1000bp, Fig. 2.2) or pTARGET for longer fragments (>1000 bp; Fig. 2.3). Ligation was performed overnight at 4°C. Ligation products were used to transform JM109 Competent Cells (New England Biolabs, Herts, UK). Transformation was achieved by adding 5 µl of ligation product to 25 µl of competent cells. The cells were then left on ice for 20 min, heat-shocked at 42°C for 45 s and returned to ice for 2 min. 200 µl of SOC medium (super optimal broth with catabolite repression) was added to the cells, which were allowed to grow at 37°C for 90 min. Cells were plated at two concentrations on Luria-Bertani broth (LB) agar plates containing ampicillin, pre-treated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside) to identify clones that had incorporated the PCR products.

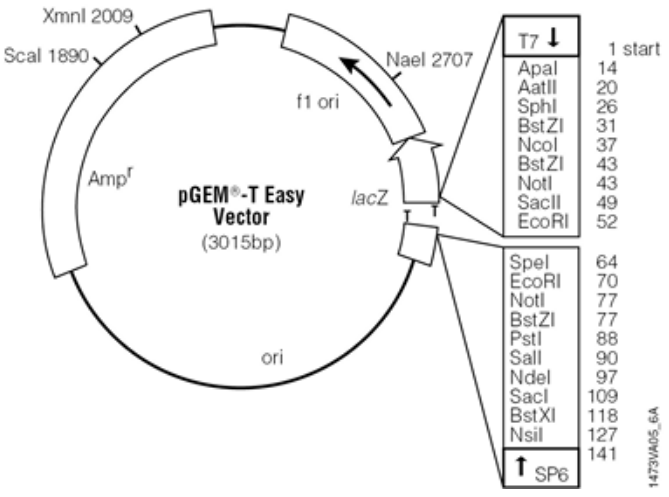


Figure 2.2 pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual, promega.com

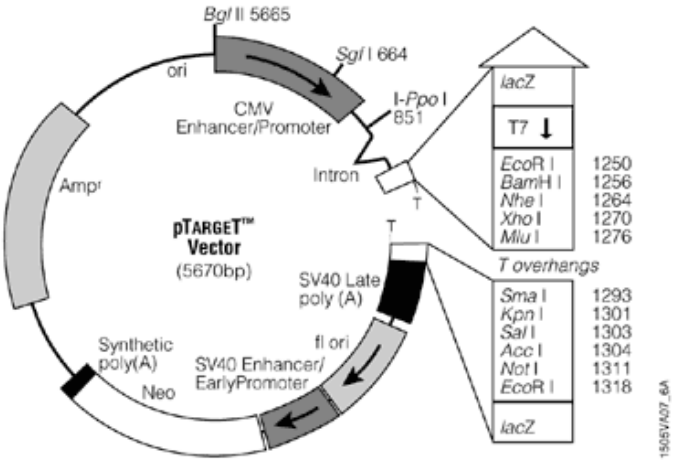


Figure 2.3 pTARGET Mammalian Expression Vector System, technical protocol from promega.com

Plates were left overnight in an incubator at 37°C. A diagnostic PCR was run for white bacterial colonies (indicating an uptake of the vector with the gene inserted) and run on 1 % agarose gel to determine the length of the inserted PCR fragment. PCR products positive for the correct fragment length were then cleaned up for sequencing.

2.5.9 **Sequencing**

2.5.8.1 For the purpose of verifying cloned and / or amplified genes corresponding to target genes and to investigate the differences between badger and commercially available ferret / dog sequences, we sequenced each gene using the Sanger sequencing method. The preparation of the samples for sequencing was a 3 step process: 1) Clean-up of the samples using salt precipitation, 2) Amplification of the specific sequence whilst incorporating a fluorescent dye, and 3) Cleaning up of the amplified sequence using another salt precipitation method.

1) *PCR clean up*: 60 µl of 20% Polyethylene glycol in 2.5 M NaCl was added to PCR products, and left to incubate at room temperature for 45 min. Plates were then spun at 2750 g for 60 min at 4°C. The contents were then emptied by inverted spinning

for 1 min at 500 g. 150 µl of ice cold 70 % ethanol was added to the wells and spun the right way up for a further 5 min at 2750 g. Contents were emptied again, as above, and the step repeated. The plate was then allowed to dry in the dark for 10 min to ensure complete evaporation of the ethanol.

2) *Sequencing reaction*: The pellet was re-suspended in 30 µl of nuclease free water. The sequencing master mix was prepared as follows (for one reaction):

- 0.25 µl BigDye (Applied Biosystems)
- 2 µl 5x dilution buffer (Applied Biosystems)
- 1.75 µl water
- 4µl of primer (10 µM diluted 1:15).

2 µl of cleaned up PCR product was added to 8 µl of sequencing master mix and put in a PCR thermocycler with the following program:

96°C for 10 s
50°C for 5 s
60°C for 1 min
} x 30

3) *Sequencing reaction clean up:* Each reaction was made up to 20 µl by adding 10 µl of nuclease-free water. 52 µl of ethanol and salt solution (7 ml of absolute ethanol mixed with 280 µl of 3 M sodium acetate, at pH 4.6) was added to each well, and the content was incubated in the dark at room temperature for 45 min. Plates were spun at 2750 g for 60 min at 4°C. The content was then emptied by inverted spinning for 1 min at 500 g. 150 µl of ice cold 70 % ethanol was added to the wells and spun for a further 5 min at 2750 g before emptying them as above. Pellets were dried in the dark for a further 10 min before being run on the sequencer.

Sequencing was performed by the Oxford University Zoology Department Sequencing facility using capillary-based Sanger sequencing run on ABI 3730xl and 3130 machines.

2.5.9.1 **Sequencing through 5' RACE products**

5'Rapid Amplification of cDNA Ends (RACE) is a method designed to amplify nucleic acid sequences from mRNA between a defined internal site and an unknown sequence at the 5' end of the mRNA. 5'RACE-ready CDNA was generated using SMARTer RACE kit (Clontech) according to manufacturer's instructions and amplified by PCR with 5'RACE primers. RACE PCR products were also cloned and sequenced as above.

2.5.9.2 **Alignments and phylogenetic trees**

Sequences were aligned with ClustalW software before checking and manually adjusting sequences if necessary, using Bioedit. These were used to construct phylogenetic trees using MEGA4 with boot-strapping (see Chapter 6 for methodological details, alignments and trees).

Chapter 3: **Repeatability of Oxidative Stress measurements**

3.1 **Introduction**

Studies of physiology are becoming increasingly applicable to the study of wild animal population ecology (Costantini 2008). Applying methods designed initially for laboratory based experiments, or those developed for other species can, however, pose unexpected technical challenges. Of particular importance is ensuring that the assays used are relevant to the enquiry being made (Horak and Cohen 2010), because in the context of natural stressors and natural genetic variation present in wild populations, assays originally developed for laboratory species and controlled conditions could be relatively uninformative.

Total antioxidant capacity (AOX) measures capacity of plasma to quench free radicals, through non-enzymatic molecular antioxidants *in vitro* (Cao and Prior 1998). Consequently, while AOX does not provide the full oxidative stress picture, it does show the *potential* to respond to an oxidative challenge (see Costantini and Verhulst 2009).

Some molecules can interfere with AOX assay results; Costantini (2011) documented the interaction between uric acid and AOX assays. Uric acid is a waste product of purine metabolism that also has antioxidant properties (Smith and Lawing 1983, Benzie *et al.* 1999). Purines are heterocyclic aromatic compounds that can be found at high levels in red meats and cereals; these include guanine and adenosine, two of the building block for DNA and RNA (Marshak and Vogel 1951). Purine metabolism is different between birds and mammals, which could influence the levels of circulating uric acid in the body. Birds, reptiles and some insects excrete their protein waste as uric

acid (uricotelics), whereas mammals metabolise it into urea (ureotelics) and excrete it that way (Munro 1969). Because uric acid is a metabolite it is unlikely to be actively regulated in response to levels of reactive oxygen species (ROS) and Constantini (2011) suggested that if AOX measurements were highly correlated with uric acid levels, such results should be corrected (by using residuals) in order to investigate life-history traits associated with oxidative stress. The level of correction, however, would be dependent on the type of assay used because of variation in biochemistry in the different AOX assays (Cohen *et al.* 2007, Costantini 2011).

Malonaldehyde (MDA) assays are one of the most commonly used biomarkers of oxidative damage (OD) because they measure the degradation product of polyunsaturated fatty acids (PUFA) a main cell membrane component (Devasagayam *et al.* 2003). Moreover, MDA is also potentially harmful to DNA and protein through a continued oxidative chain reaction (Halliwell and Gutteridge 2015).

Measuring resistance to oxidative stress, or the integrity of cell membranes, can also be done by assessing the half-life of red blood cells (RBC) in the presence of free radicals *in vitro*. This assay can be run in two different formats, either by using full blood in the assay, or by using isolated RBC. Despite the fact that RBCs contain extensive antioxidant defence systems, their membranes are relatively susceptible to cell membrane damage through peroxidation and are thus thought to provide an estimate of resistance to oxidative stress (Reddy *et al.* 2007). Additionally, RBCs circulate for approximately 110 days (Klinken 2002), giving an indication of the RBCs' history in the past 3 months.

As previously mentioned, hydrogen peroxide (H_2O_2) is one of the more stable ROS molecules, and is thus one of the easier ones to measure. H_2O_2 is generally produced by a range of oxidation enzymes and is involved in signalling. There are several assays available, that with very little modification can be used either for the measurement of H_2O_2 (Pick and Keisari 1980, Mohanty *et al.* 1997), or for the measurement of peroxidases, which neutralise H_2O_2 (see Chapter 2).

Oxidative stress, an imbalance of reactive oxygen species (ROS) and antioxidants leading to oxidative damage affecting proteins, DNA, lipids and polysaccharides (OD; Sies 1991), is of key interest in eco-physiology (Monaghan *et al.* 2009). However, although there are many different commercially available assays to measure the different molecules involved in the OS / OD chain, choosing the right indicators to monitor OS can be difficult. Indeed, most assays have been developed for biomedical studies, where access to animals is guaranteed (as opposed to having to trap them) and environmental conditions are unvarrying. Thus most methods need to be validated or modified for ecological studies (see Costantini 2011). Additionally, ROS are difficult to measure accurately because of their high reactivity and resulting short ½-life (e.g. superoxide anions: Degli Esposti 2002), although some ROS are easier to measure, such as hydrogen peroxide which is more stable. Antioxidant systems, on the other hand, are comprised either of a molecular component produced endogenously, such as glutathione, bilirubin, uric acid and vitamin C (Stocker and Frei 1991), or of a dietary component, such as vitamins A and E, or thiols (Bouayed and Bohn 2010), in conjunction with an enzymatic component (Harris 1992).

The validation of these assays for the use in wild mammal species requires a model species which is sufficiently large to take blood volumes sufficient for a variety of OS assays, as well as the option for repeat sampling in order to assess repeatability of these assays (i.e. consistency of results to discriminate between desired measurability of long-term / natural OS vs short-term handling-induced "artificial" OS).

Indeed, in laboratory conditions, it is easy to access individual X on day Y. In wild populations, the chances of trapping a particular individual on a specific day are relatively slim. For this reason, it is important to have assays which will not be sensitive to this potential differential in capture dates. While ability to mount a short and intense response to pathogens, or ROS, could be important for wild animals, in the context of seasonal studies or yearly variations, it is critical to have markers that will be representative of a longer time period. It is thus preferential to use markers that could be informative on OS levels regardless of when the animals were caught within a certain time-frame.

When sampling wild animals, even as part of an established mark-recapture regime, interval of recapture is generally too unpredictable or infrequent to measure short-term and cyclical variations in OS biomarkers (e.g., daily or weekly variations). The badger trapping protocol in Wytham involves two-week trapping sessions per season (with subsidiary trapping, if required; Macdonald *et al.* 2015a), and while recaptures within sessions are generally released without re-processing (no need for duplicate samples & measures), it is possible to recapture and sample badgers within a shorter period of time (1day – 2weeks). High recapture rates in this badger study allow for such short-term re-sampling, enabling intra-individual variability in assays to be established over the

course of days (see Noonan *et al.* 2015b). This enabled the most stable markers over short periods to be chosen for further research.

- The first objectives of this chapter were to identify what level of correlation occurs between AOX and UA measurements, to inform the decision of whether AOX needs to be corrected for UA in badgers.
- The second set of objectives involved the stability of OS biomarkers. Badgers were deliberately recaptured and resampled in order to assess the stability of oxidative stress biomarkers over short time periods and choose the most robust and reliable measures in order to actually be measuring seasonal / yearly variations rather than daily / short term changes.

3.2 Methods

Using a long-term study of badger socio-ecology in Wytham Woods, UK (details in Chapter 2), plasma, full blood and red blood cell (RBC) samples were collected for 181 adult badgers. In order to assess the stability of OS biomarkers, 20 individuals were recaptured and deliberately resampled over the course of the two weeks of trapping period.

3.2.1 Plasma assays

The AOX assay I used is based on a method similar to one that produced highly correlated results with uric acid measurements in birds (FRAP assay, Costantini 2011). Therefore, uric acid (UA) was also measured in all of samples. For technical details on how the assays were performed, see Chapter 2.

For the assessment of stability of OS biomarkers, a series of assays were run on the 20 individuals that had been recaptured.

Plasma was also assayed for AOX to determine the capacity of individual's plasma to neutralise free radicals (see Chapter 2). UA was measured in order to quantify its contribution to AOX (Chapter 2). H_2O_2 was measured to investigate ROS production (see Chapter 2), lipid peroxidation was measured as malonaldehyde (MDA) using the thiobarbituic acid reactive species (TBARS, see Chapter 2) assays, in order to quantify oxidative damage and RBC ½ life: an assay where full blood or isolated RBCs are subjected to an oxidative environment, and the time until half the RBC have lysed is calculated. This assay was run twice, once using full blood and once using isolated RBC only. For details see chapter 2.

3.2.2 Statistical analyses

3.2.2.1 Total antioxidant capacity (AOX) vs Uric Acid (UA)

The correlation between AOX and UA in 181 adult individual badgers sampled during 2012 was calculated using Pearson's correlation test ('stats' package in R).

3.2.2.2 Stability of OS biomarkers

In order to assess the stability of biomarkers, linear mixed models were run using the lme4 package in R, with the first capture measure as the outcome variable, and the second capture measure as the explanatory variable. Badger ID was included as a random effect to account for pairing of the data. RBC, AOX and LP were log transformed to meet the assumptions for linear mixed models.

3.2.2.3 **Inter-correlation of OS variables, Principle component analysis**

In order to investigate the correlation between OS variables (AOX, LP, PER, RBC), a principle component analysis was run using the package prcomp in R.

3.3 **Results**

3.3.1 **Total antioxidant capacity vs uric acid**

AOX and uric acid were positively correlated (r= 0.33, p<0.001, n=181, Fig. 3.1), with uric acid levels being generally lower than AOX levels, as uric acid is only one of the many antioxidants present in the body.

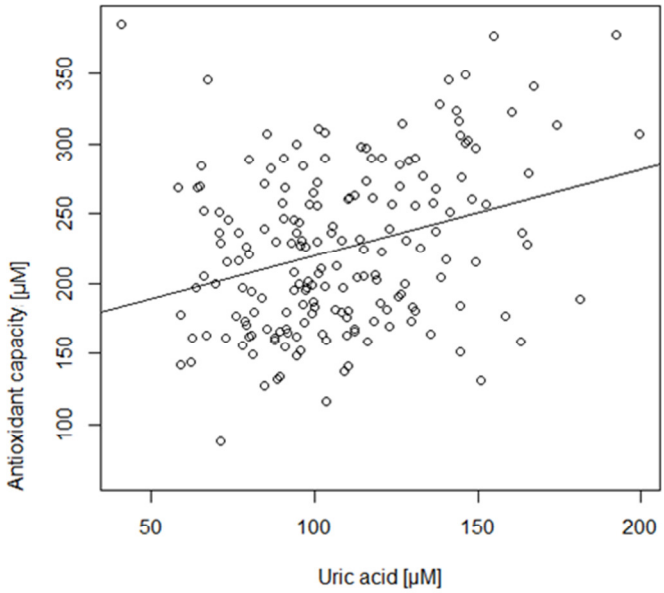


Figure 3.1. Positive correlation between uric acid and total antioxidant capacity.

Samples were run for n=181 badgers throughout the year of 2012

3.3.2 **Stability of OS biomarkers over short-term re-sampling**

Lipid peroxidation (Fig. 3.2) and RBC ½ - life (Fig. 3.3) were the only markers significantly associated between recaptures, with antioxidant capacity being marginally non-significant (Fig 3.4). Model values for all measurements are presented in Table 3.1.

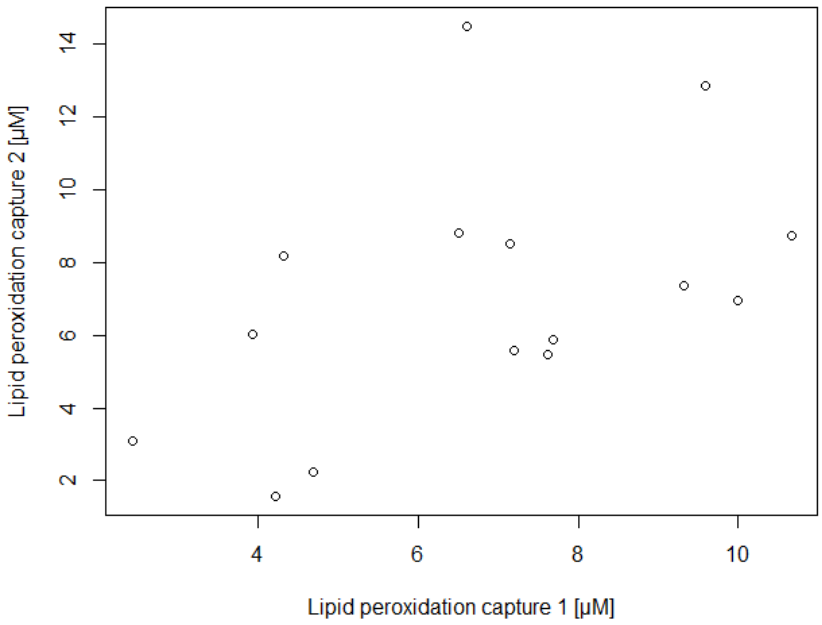


Figure 3.2 Positive correlation of lipid peroxidation between first and second capture.

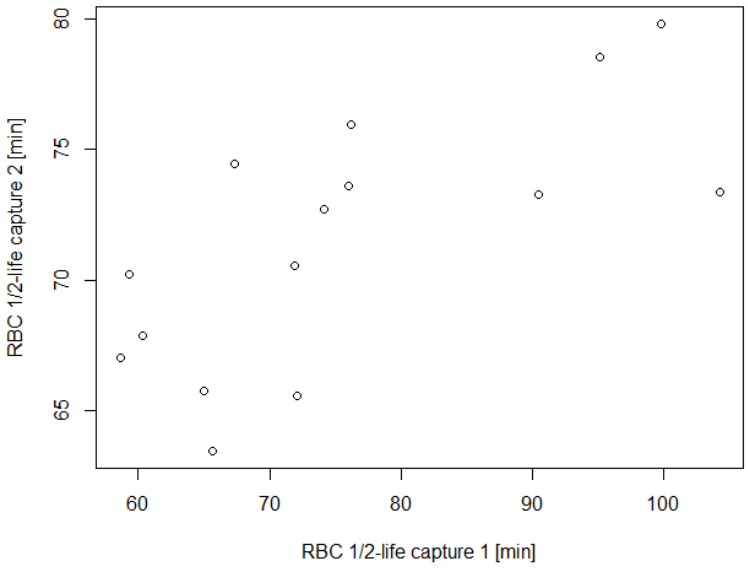


Figure 3.3. Positive correlation of RBC ½-life between first and second capture, using isolated RBCs.

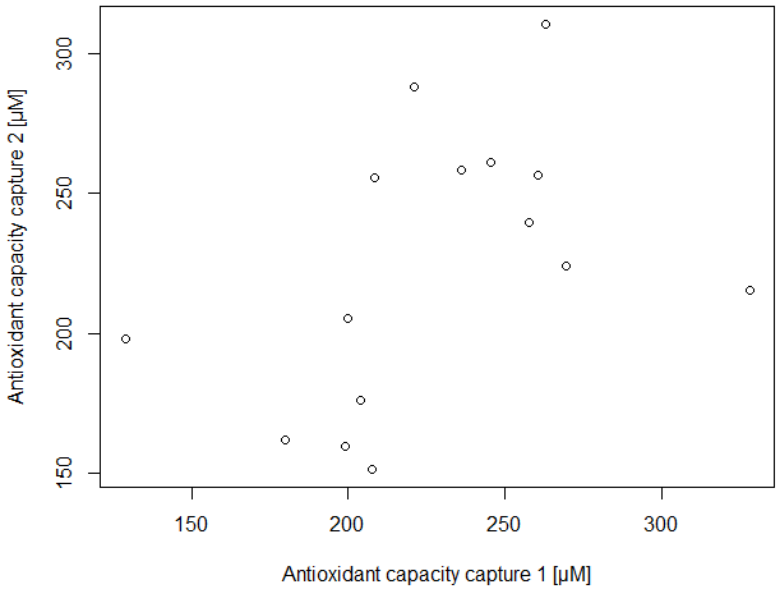


Figure 3.4 Correlation of AOX between first and second capture.

Table 3.1. Summary of statistical models between assays for the first and second capture over a 2 week time frame.

	θ	Lower 95% CI	Upper 95% CI
Log (AOX) intercept	3.11	0.17	6.06
Log(AOX)	0.43	-0.06	0.98
Log(LP) intercept	1.01	0.40	1.61
Log(LP) recapture	0.46	0.16	0.78
Log(RBC) intercept	-2.85	-6.74	1.03
Log(RBC) recapture	1.68	0.77	2.59
H_2O_2 intercept	0.20	0.06	0.34
H_2O_2 recapture	0.05	-0.42	0.52
UA intercept	112.98	56.52	169.78
UA recapture	-0.04	-0.59	0.50
FB intercept	77.78	27.19	128.37
FB recapture	0.04	-0.57	0.64

3.3.3 **Principle component analysis**

Principle component analysis did not show clear patterns of correlation between the 4 OS variables. Indeed, it takes 3 principle components (PCs) to explain 85% of the variance in the OS variables (Table 3.2).

There are strong negative loadings of AOX, LP and to a lesser extent RBC on PC1. There is a strong positive loading of RBC and a strong negative loading of PER on PC2, with a lower positive loading of AOX. There is a strong negative loading of LP and a strong positive loading of PER on PC3 with a lower positive loading of RBC.

Table 3.2 Summary of the first three principal components of OS measurements

Variable	PC1	PC2	PC3
Eigenvalue	1.39	1.24	0.77
% of variance explained	34.63	30.84	19.34
Cumulative % of variance explained	34.63	65.48	84.81
AOX	-0.65	0.33	0.04
LP	-0.64	-0.18	-0.58
PER	-0.16	-0.55	0.73
RBC	-0.39	0.75	0.37

3.4 Discussion

3.4.1 Total antioxidant capacity (AOX) vs uric acid (UA)

This study confirmed a correlation between AOX and UA in badgers, although the degree of correlation between AOX and UA was considerably less than the correlation found previously in birds by Costantini (2011) using a similar test (correlation = 0.91). The difference in these results is likely to be due to the difference in uric acid metabolism between birds and most mammals (excluding hominids; Varela-Echavarría *et al.* 1988). Mammals have a functional uricase gene coding for an enzyme (urate oxidase) that further degrades uric acid into the more soluble allantoin. This gene is absent in birds, most reptiles and hominids (Varela-Echavarría *et al.* 1988, Oda *et al.* 2002). Consequently the accumulation of uric acid will be much lower in mammals

than in birds, resulting in a lower ratio of uric acid to other antioxidants. Comparing results here with zebra finches (Costantini 2011), uric acid levels were 10 times lower in badgers. Despite the positive correlation, uncorrected antioxidant capacity provides an acceptable technique to measure badger antioxidant capacity because of the relatively small contribution uric acid makes to their total antioxidant capacity. This decision was also based on the fact that, despite the lack of active uric acid regulation (as a waste product), it still has antioxidant properties and contributes to the overall antioxidant capacity (Becker 1993).

3.4.2 **Stability of oxidative stress biomarkers over short-term recaptures**

In order to draw conclusions from experimental studies, it is important to be confident that the methodology is appropriate and accurate. In this study, different assays exhibited both high and low extents of intra-individual variability over short periods, making some of them unsuitable for work on seasonal variations. Below every assay described in the methods and results will be discussed individually for suitability for the rest of the study. Unfortunately, due to sampling volumes required for repeat measurements, it was not possible to carry out these analyses on cubs, and therefore conclusions have been assumed from adult values.

3.4.2.1 **Total antioxidant capacity**

Circulating levels of non-enzymatic antioxidants were not significantly associated between first and second capture, although the result was bordering on significance. Because of the marginality of the result, and the fact that other assays were not financially viable in this study, the antioxidant capacity assay will be continued for the duration of the study. .

3.4.2.2 **Uric acid**

UA values did not correlate between short-term recaptures. This accords with the passive process by which uric acid is produced, as an end product of purine metabolism rather than as a targeted antioxidant (Cohen *et al.* 2007).

3.4.2.3 **Hydrogen peroxide (H_2O_2)**

Despite H_2O_2's relative stability compared to other ROS, it still tends to undergo rapid decomposition into water and oxygen (Cohen and Hochstein 1963), making it difficult to measure. The lack of correlation between one measurement and the subsequent recapture is thus not entirely surprising, as circulating levels of H_2O_2 will rapidly reflect changes in cellular production. Additionally, results from this study show extremely low levels of H_2O_2 (< 1 µM) compared to those listed in the published literature for mice (1-5 µM; Banerjee *et al.* 2003). It is therefore unlikely that the low levels of H_2O_2 found here resulted from sample degradation during storage, as they were stored in accordance with standard assay protocols and defrosted slowly on ice.

The combination of low and rapidly changing production of H_2O_2 could explain the lack of a correlation between samples of recaptured animals in the current study. This suggests that H_2O_2 does not provide a suitable measure of OS for investigating seasonal changes. From a biological perspective, it is intriguing that badgers have lower levels of H_2O_2 than other mammals (e.g. mice; Matoba *et al.* 2000). This could suggest a high level of antioxidants circulating that could quench H_2O_2, or simply a lower production of H_2O_2 in general.

3.4.2.4 **Lipid peroxidation (MDA)**

Oxidative damage to lipids was significantly correlated between re-captures of individuals. Measuring MDA using the thiobarbituic acid reactive species as in this

study has been criticised in the past (Del Rio *et al.* 2005), because lipid peroxides can cause self-propagating chain reactions which can artificially increase MDA levels in the respective sample during the assay itself. For this reason, to minimise the further peroxidation of lipids while running the assay, I added the antioxidants to the sample before the assay was run, following the method suggested by Oakes and Van der Kraak 2003). An additional element, with potential to compromise the assay, is that dietary intake of PUFA's could lead to increased circulation of MDA, which does not reflect actual damage to cell membranes. This study demonstrated, however, that while absolute values of MDA must be used with caution, the comparison between individual levels of MDA from this assay still provides an informative index of an individuals' levels of OS.

3.4.2.5 **Red blood cell killing assay –RBC only vs full blood**

The RBC killing assay, using RBCs only, was the assay that showed the highest fidelity between recaptured individuals. The long circulation time of RBCs (Klinken 2002) makes them ideal measurements of resistance to OS in studies where tissue samples are not accessible, such as ecological monitoring and conservation studies.

When running the assay using full blood (as opposed to isolated RBC), recapture data showed high intra-individual variability. In the assay with full blood, the red blood cells benefit from protection by antioxidants present in the plasma, thus being more likely to reflect how RBC would fair *in vivo*. There are many confounding factors that can make variation in this assay more difficult to interpret than with RBCs alone. Indeed, not only does this assay examine RBC resistance, it also provides a measure of plasma antioxidant capacity. The amount of RBCs present in the full blood sample is also strongly affected by hydration levels, which can cause the ratio of RBC to plasma (and

thus antioxidant capacity) to vary substantially (Senay and Christen 1965). These results make the RBC version of the assay much more suitable as an OS biomarker for observation of seasonal and yearly variations.

3.4.3 **Principle component analysis**

The principle component analysis did not allow me to replace the OS variables with fewer, strongly influencial principle components. As such, these will not be used in the following chapters, but instead the individual variables (AOX, LP, RBC and PER) will be used.

3.5 **Conclusion**

The use of accurate measurements to study OS related to life-history is an important factor in planning experiments. In this chapter it is clear that the methodology developed for and investigated in birds, needs to be validated, and potentially adapted, for mammal field research. Additionally, even the most precise measurements and kits can be uninformative when used in the wrong context, as shown with the hydrogen peroxide assays in this chapter. Investigating seasonal changes of a metabolite which has a very short half-life in the blood is likely to provide mainly spurious results. By using recapture data to validate assays, this chapter provides evidence that appropriate assays (AOX, lipid peroxidation and RBC ½-life) are used in the next chapters.

Because the decision was made to not correct AOX for UA in this study, as uric acid is a much smaller component of AOX in mammals than in birds. UA measurements were subsequently discontinued. The H_2O_2 assay was also deemed too variable, it was discontinued after 2012 and the remaining kits were used to assess peroxidase levels

(antioxidant enzymes) instead (see Chapter 2). The full blood version of the RBC ½-life assay was deemed too variable as well and was discontinued.

Based on the stability of OS markers described above, it was decided that in the next chapters OS would be measured using AOX, MDA and the isolated RBC version of the RBC ½-life assay. Peroxidase levels will complete this group of assays. I propose that these methods are adapted to long-term ecology studies of mammals.

Chapter 4: **Age-related changes in oxidative stress correlates in the European badger: juvenile survival, trade-offs and reproduction.**

4.1 **Abstract**

Oxidative stress, i.e. an imbalance between pro-oxidants and antioxidant defences, can lead to severe acute and chronic tissue damage, with implications for life-history trade-offs. Cub body condition and lipid peroxidation levels were linked to cub survival probability. Among surviving cubs, I observed a trade-off such that individuals investing more in antioxidant defences attained overall shorter adult foot size, indicating less investment in skeletal development. Furthermore, among surviving cubs, the ultimate adult body-length an individual attained (accounting for sexual dimorphism) was shorter, when cubs had lower body condition.

We also found age-related differences in oxidative stress biomarkers between cubs, prime age adults (1-5 yrs) and old adults (6+ years), where cubs exhibited significantly greater oxidative damage than did adults of any age. Cubs had lower antioxidant capacity than did adults in the spring, which subsequently converged upon higher adult levels as individuals mature. Despite using a longitudinal approach, individuals showed no changes in antioxidant defences or oxidative damage with increasing age. I did, however, find that levels of oxidative damage were significantly higher in females that had reproduced that year than in non-reproducing females.

This finding illustrates that investment in antioxidant defences and levels of oxidative stress can be traded-off with traits such as first year growth, before reaching adult levels; although the measures of oxidative damage used here show that this is not

necessarily cumulative for individuals even when reproduction causes heightened oxidative stress.

4.2 **Introduction**

Wild animals are subject to a range of stressors (e.g., diseases, food stress, reproduction, intra- and inter-specific competition: Connell 1983, Pedersen *et al.* 2007, Higginson *et al.* 2014, Lukas and Clutton-Brock 2014, Newsome *et al.* 2015), and the relative effects of these stressors on health, development and survival can differ with life-history stage (e.g. Rayor and Uetz 1993, Clark *et al.* 2014). Although causing effects apparent at the whole organism and organ-system levels (Pottinger *et al.* 2002), these substantial physiological costs (e.g., Metcalfe 1986, Speakman 2008) ultimately exert their pathology through the cellular processes (Costantini and Bonadonna 2010) of oxidative stress (OS; Metcalfe and Alonso-Alvarez 2010). As organisms attempt to compensate stress, elevated metabolic rates increase the production of reactive oxygen species (ROS), which need to be quenched by antioxidant systems (many of which are nutrition dependent). Failure to neutralise ROS causes oxidative damage to proteins, DNA, lipids and polysaccharides (Halliwell and Gutteridge 2015), with implications for somatic maintenance and reproductive success. Furthermore, because ROS are also key factors in cell signalling and immune reactions they cannot be eliminated entirely (Bogdan *et al.* 2000, Thannickal and Fanburg 2000); instead there is an important redox balance that must be maintained to ensure functionality while minimising toxicity (Rhee 2006). Finding and maintaining this balancing point can be particularly challenging for wild animals, where the environmental, social and pathogen-driven stressors that drive oxidative stress can have unpredictable and often severe impacts on their survival and reproductive fitness (Monaghan *et al.* 2009, Metcalfe and Alonso-Alvarez 2010). As previously mentioned, oxidative imbalance leads to oxidative

damage, the measurements of which can be varied. Lipid peroxidation reflects damage done in particular to cell membranes, through oxidation of poly-unsaturated fatty acids (Selman *et al.* 2012).

Investment in antioxidant defences and resistance to oxidative stress are clearly critical, but costly, and, according to the disposable soma hypothesis (DSH: Kirkwood and Rose 1991), are traded-off against other physiological and developmental traits, as well as reproduction (Monaghan *et al.* 2009). These trade-offs depend on various intrinsic factors such as age, sex and individual genetics, as well as environmental conditions (see Chapter 5), where free radicals are implicated as a key factor in ageing and senescence (Free radical theory of Ageing, FRTA, Beckman and Ames 1998, Ashok and Ali 1999, Sohal and Orr 2012).

4.2.1 **Life-history interactions with OS**

While ageing is a by-product of natural selection, longevity is an adaptive mechanism (Sinclair 2002). Longevity assurance mechanisms (LAMs) are a corollary of the DSH, where these mechanisms provide an optimal compromise between somatic maintenance and reproduction (Kirkwood and Austad 2000). The majority of longevity genes seem to favour somatic maintenance in harsh environmental conditions, and reproduction in good conditions (Schumacher *et al.* 2008, Gems and Partridge 2013). Investment in somatic maintenance will also depend on the reproductive strategy of each species. For species with high risk from extrinsic mortality factors (predation, disease, etc), age at first reproduction and investment in offspring will be higher than investment in somatic maintenance; termed r-selected species by Pianka 1970). K-selected species, on the other hand, with lower risk of extrinsic mortality, invest more substantially in longevity

in order to maximise lifetime reproductive success (LRS; Reznick 1985, Merilä and Sheldon 2000).

Particularly in K-selected species, age-dependent trade-offs occur principally through the differential demands faced by functional age-classes. While adults seek to maximise LRS, juveniles must first grow and survive to the point where they are reproductively active. Despite early investigations of ageing suggesting that senescence was not a major factor for wild populations - due to individuals dying from other causes prior to senescence (Reznick *et al.* 2004, Williams *et al.* 2006) - there is increasing evidence that senescence does affect the dynamics of wild populations (Mysterud *et al.* 2001, Bouwhuis *et al.* 2009).

4.2.2 **Impacts of OS on juvenile development and survival**

Although ROS production and antioxidant systems vary throughout life (Papa and Skulachev 1997, Metcalfe and Alonso-Alvarez 2010Metcalfe and Alonso-Alvarez), juveniles are particularly vulnerable to OS, and thus OD because they start with under-developed antioxidant systems. Juveniles generally also experience constraints on food availability, either due to subordinate social status (Fell *et al.* 2006), compromising access to food compared to adults (Maclean and Metcalfe 2001), or their greater vulnerability to predators compromising foraging activity (Kotler *et al.* 1991, Verdolin 2006).

In addition, juveniles rely substantially on their innate immunity, until their acquired immune system develops through exposure to pathogens (Good and Papermaster 1964), where innate immune responses to infection generate ROS (Bogdan 2000). This places

juveniles at greater risk of disease and pathogenesis (Newman *et al.* 2001, Day 2007), further exacerbating the generation of ROS.

Starvation and / or malnutrition in tandem with disease can thus limit a juvenile's ability to acquire and absorb micronutrients, essential for its antioxidant defences, immunity and normal metabolic function (Klasing 1998, Bhaskaram 2002). Consequently, the rate of development of antioxidant competency must be traded off against the high metabolic demands of growth subject to restricted and compromised energy budgets (Stevens *et al.* 2000).

4.2.3 **Impacts of OS on reproductive success**

At sexual maturity, the drive to reproduce adds to life-history stressors. Sex hormones can exacerbate OS levels (Vina *et al.* 2005, Alonso-Alvarez *et al.* 2007a), where testosterone is associated with immune-suppression in males (Aitken and Baker 2004; badgers: Buesching *et al.* 2009), and unquenched ROS can impair spermatogenesis (Sikka 2001). In female mammals, OS can impair ova production (Lector 1996), and gestation and lactation generate substantial ROS (Agarwal *et al.* 2005); indeed, lowered probability of post-reproductive survival has been linked OS levels in birds, reptiles and mammals (Bize *et al.* 2008, Isaksson *et al.* 2011b).

4.2.4 **Impacts of OS on senescence**

OD is also cumulative, where repeated or chronic OS can lead to cellular senescence, causing mitochondrion and the electron transport chain to become less efficient and leak more ROS as individuals age (Beckman and Ames 1998). Consequently, older individuals of many taxa exhibit higher levels of ROS, lower antioxidant capacity and

higher levels of oxidative damage (Martin and Grotewiel 2006, Monaghan *et al.* 2008, Ricklefs 2008), placing them at higher risk of geriatric disease (Ricklefs 2008).

4.2.5 **Model Species: The European badger**

Many studies have investigated lifetime OS responses and OS trade-offs in wild birds (see Costantini 2008), although studies in wild mammals are fewer and tend to be cross-sectional (Nussey *et al.* 2009). The European badger (henceforth, 'badger') is a medium sized, relatively long lived K-selected mammal with no extant predators in the UK. These traits, combined with its tractability and the wealth of knowledge available from several long term studies (Kruuk and Parish 1987, Rogers *et al.* 1997, Byrne *et al.* 2012, Macdonald *et al.* 2015a) make it an informative model species for investigating age-dependent OS responses and trade-offs. Capture-mark-recapture studies offer the opportunity to repeat sample individuals at regular intervals, and provide longitudinal datasets from which cub development and survival metrics can be extracted, along with evidence of adult ageing / senescence trends (e.g., Buesching *et al.* 2009, Dugdale *et al.* 2011b). Because OS balance is comprised of several factors (ROS, AOX and OD; Monaghan *et al.* 2009), it is insufficient to investigate only one biomarker of the OS system. Instead, it is necessary to look at both the AOX's ability to quench ROS as well as the extent to which this mitigates OD. Measuring ROS can be difficult due to the high reactivity and short half-life of these molecules, often leading to this measurement being "assumed" from the difference between antioxidant capacity and oxidative damage.

Aside from simple practicality, badger life-history traits have been linked to OS; namely, antecedence of antioxidant defences in juveniles (Montes *et al.* 2011, Bilham *et al.* 2013), especially in years with challenging environmental conditions. To assure

continued survival until an age when breeding is possible, cubs must first invest in physiological systems that maximise likelihood of surviving to maturity. In our study system (Wytham Woods, Oxfordshire), 50% of cubs die by age 2 years, and of those surviving to sexual maturity, 50 % of these die by age 5 years; however, badgers can live to be more than 15 years old, although only ca. 10% of individuals will live past 8 years of age (Macdonald *et al.* 2015a).

- One of my primary aims here is to investigate whether badger cubs trade-off somatic growth (body length, body condition) during development against antioxidant defence. Additionally I aim to establish if oxidative damage limits survival in cubs.
- A cross sectional analysis of age-classes will investigate whether cubs have different levels of OS biomarkers compared to prime-aged or old adults.
- Our second aim is to establish whether reproduction increases oxidative damage or decrease antioxidant defences in females. In badgers, litters of 1-4 cubs (mean 1.48) are produced by 30% of mature females each year – established from genetic pedigree (Annavi et al. 2014). Reproductive capacity and breeding status peak around 5 years old for males and 3 years old for females, before declining with age (Dugdale *et al.* 2008, Buesching *et al.* 2009).
- Our final aim develops Bilham *et al.* (2013)'s preliminary finding that older individuals had lower antioxidant capacity than did cubs and prime aged adults. Building on this, I investigate whether antioxidant capacity decreases and OD increases as individuals age, potentially providing a mechanistic explanation for the changes in reproductive success.

4.3 Methods

Badgers were sampled as part of an ongoing socio-ecological study in Wytham woods, UK (see Chapter 2 for details). Briefly, badgers were caught seasonally (spring, summer, autumn), sedated, measured, weighed and sampled for blood. Measurements taken included head-body length, zygomatic arch width, and length of both rear pasterns (foot length). Body condition (BCI) was calculated as $\log_e$(length)/$\log_e$(weight) (Noonan *et al.* 2014).

Several complementary techniques were applied to measure OS, including total antioxidant capacity (AOX), peroxidase enzymatic activity (PER), red blood cell half-life (RBC ½-life; which measures resistance to OS as the capacity of cell walls to respond to an oxidative challenge). In addition, OD was measured as lipid peroxidation (LP). All techniques are detailed in Chapter 2. Samples were analysed for a total of 568 samples (422 adult and 146 cub samples) for 280 individual badgers (of which 102 were cubs) over spring, summer and autumn 2012-2014 (only spring and summer for 2014). Samples were chosen in order to follow individuals vertically, including cub development and adult senescence (i.e. I needed individuals which had been captured and sampled multiple times over the 3 year process).

4.3.1 Impacts of OS on juvenile development

4.3.1.1 Cub survival and OS

According to estimates for this focal study population, approximately 23 to 30.5% of badgers are likely to die underground, prior to being trapped and recorded from late-May onwards (aged ca. 12 weeks; Macdonald *et al.* 2015a), and thus all analyses are implicitly restricted to a subset of cubs that have already survived until weaning. From

this known subset of individuals, recapture data were used to quantify subsequent survival rates through the summer, autumn and winter, to reach maturity in the following year. If a cub was not recaptured within 2 years, its 'date of death' was allocated as being shortly after its last capture. Using this method, estimates were derived for the number of cubs that died between spring and summer trappings (giving an indicator of "summer survival" probability as a binomial variable), what proportion of cubs died between summer and autumn trappings (giving "autumn survival"), and what proportion of cubs died over their first winter (giving "survival to 1 year" – Table 4.1).

Models for survival, at each time period, were built to include cub BCI, sex, and all OS metrics as fixed effects. Small sample size precluded the inclusion of RBC ½-life in these survival analyses. All variables were mean centred prior to analyses in order to satisfy the assumptions of the model (homoscedasticity and normality of distribution). Differences in survival rates throughout the year were accounted for by including season and year as random effects.

Survival until summer = f (sex + BCI+AOX + log(LP) + PER) + (Season +Year)

Survival until autumn = f (sex + BCI+AOX + log(LP) + PER) + (Season +Year)

Survival to 1 year = f (sex + BCI+AOX + log(LP) + PER) + (Season +Year)

Table 4.1. Cub cohort survival rate throughout years and seasons, for individuals that were of a body-size that allowed sedation and blood sampling

	2012	2013	2014	Total
Died spring-summer	15	7	17	**39**
Died summer-autumn	3	5	5	**13**
Died autumn-1 year	1	4	1	**6**
Survived to 1 year	20	9	15	**44**
Total	**39**	**25**	**38**	**102**

4.3.1.2 **Cub oxidative stress and effects on adult size**

To investigate the effects of OS and body condition on the ultimate adult size (head-body length, zygomatic arch width, foot length) each of the 44 individuals surviving to maturity attained, the following models were tested:

Head-body length = f (sex + BCI+AOX + log(LP) + PER) + (1|Season) + (1|Year)

Zygomatic arch width = f (sex + BCI+AOX + log(LP) + PER) + (1|Season) + (1|Year)

Foot length = f (sex +BCI+ AOX + log(LP) + PER) + (1|Season) + (1|Year)

Because the small number of cubs that survived to adulthood (n=44) precluded a model selection approach for these analyses, a Bayesian approach via the *R* package *MCMCglmm* was used (Hadfield 2010). Here, multiple iterations with re-sampling better accounted for this smaller sample size than a model averaging approach (Ver Hoef and Boveng 2015). Following Hadfield (2010), an uninformative, inverse-gamma prior distribution was used. The number of iterations, thinning interval and the burn-in period were determined using diagnostic tests in the *R* package *coda* (Plummer *et al.* 2006).

Seasonal changes in BCI between age-classes were investigated using analysis of variance (ANOVA, *stats* package in R).

Previous analyses suggested antecedence of cub antioxidant capacity (Bilham *et al.* 2013), posited to arise to enable immunologically immature cubs to deal with substantial early exposure to ROS (due to energetic demands, weather condition (see Chapter 5), parasite levels etc).

Furthermore, I sought to establish whether distinct phenotypes exist within cub cohorts, trading-off antioxidant capacity against other developmental parameters in ways subject to providing a selective advantage in years of high OS; although potentially a selective disadvantage in benign, low ROS, years.

I therefore applied an information-theoretic approach (Burnham & Anderson 2010) to establish the most parsimonious (i.e., the most supported) model explaining any interaction between OS measurements and age-class, in relation to BCI, sex and season. Social group was also included as a random effect in order to account for the potential effects of increased relatedness and social interactions. Badger identification number (badger ID) was included as a random effect to account for repeat sampling of individuals. Badger ID was nested within social group. Year was included as a random effect, due to the difference in mean cub cohort survival rate between years (Table 4.1), where annual cub cohort survival rate provides a proxy for the overall strength of stressors (mortality factors) operating in that year (e.g., inclement weather; see Chapter 5). Lipid peroxidation data, RBC and PER were log-transformed to satisfy the assumption of the model. AOX was square rooted for the same reasons. Generalised

linear mixed models were built using the lme4 package. Models with a ΔAIC of less than 4 were averaged using the MuMIn package.

From these variables, models were built to explain each measurement in the OS balance, with or without interactions (age-class* BCI, age-class * season). Per OS metric global models were:

Antioxidant capacity = *f* (BCI* age-class + age-class*season + sex) + (1|Social Group/ BadgerID) + (1|Year)

Peroxidase = *f* (BCI* age-class + age-class*season + sex) + (1|Social Group/ BadgerID) + (1|Year)

Lipid peroxidation = *f* (BCI* age-class + age-class*season + sex)+ (1|Social Group/ BadgerID) + (1|Year)

RBC ½-life = *f* (BCI* age-class + age-class*season + sex)+ (1|Social Group/ BadgerID) + (1|Year)

For each model AICc (AIC adjusted for small sample size in relation to the number of parameters, where a smaller value indicates stronger support for the model) was derived and the relative weight of each model (W) was calculated. Model averaging was then applied for models with a ΔAIC < 4 to determine a weighted average of the predictions for each of the models, and their associated 99% confidence intervals; accounting for uncertainty in model selection. The predicted values (Θ) from each model are dependent both on the magnitude of parameter estimates and on the relative weight (*W*) of each model including that parameter. Significant predictors for OS values were those

for which 99% confidence intervals for the mean weighted parameter estimates did not overlap zero. 99% confidence intervals were used as a more stringent cut off point to avoid false positives from multiple testing.

4.3.1.3 **Female reproduction and OS**

Because parturition occurs underground, and cubs remain within the sett for the first 8 weeks of their lives (Macdonald *et al.* 2015a) successful reproduction in females was assigned based on evidence of lactation. Following Dugdale *et al.* (2011a), lactation was inferred from teat length and diameter, as well as the presence of a bald patch surrounding the teats due to suckling. In the absence of genetic pedigree analysis, litter size was not known.

Spring OS and OD (LP) metrics for females that had not lactated were compared to those that had using t-tests. Again, lipid peroxidation measurements were log-transformed in order to adhere to assumptions of normality.

4.3.1.4 **Impacts of OS on senescence**

In order to investigate if individual age interacts with OS levels (i.e. increasing susceptibility to OS / OD with advancing age), models were built that included age as a continuous variable (1-12 years) along with its interaction with sex. Badger ID was included as a random effect to account for the longitudinality of the data (Ericsson *et al.* 2001, Reid *et al.* 2003), nested within social group as above.

Antioxidant capacity = f (Age*sex) + (1|Social Group/ BadgerID)

Peroxidase = f (Age*sex) + (1|Social Group/ BadgerID)

Lipid peroxidation = f (Age*sex) + (1|Social Group/ BadgerID)

RBC ½-life = f (Age*sex) + (1|Social Group/ BadgerID)

As previously, AICc values were computed, and model averaging was applied to models with a ΔAIC < 4 Again, 99% CI were used as a more stringent cut off to avoid false positives from multiple testing.

4.4 **Results**

4.4.1 **Oxidative stress and cub survival**

No evidence was found of any metric of OS constraining individual survival probability between spring and summer; only body-condition was influential on survival over this period. That is, the fatter an individual was in spring, the more likely it was to survive until summer. There was, however, some support among the surviving sub-sample of individuals for an effect of LP (oxidative damage) on probability of cub survival from summer to autumn, and from autumn through the winter (i.e. through their first year), although 95% CIs marginally overlapped zero (Table 4.2 and 4.3). Body condition ceased to have any influence on cub survival from summer onwards (Table 4.4).

Table 4.2 Survival through until summer, model variables

Coefficient estimates (β) are presented along with their 95% confidence intervals (CI). Asterisks denote variables that differed statistically from zero (based on 95% CIs). Note $5x10^6$ iterations, with $1x10^3$ burn-in and thinning interval of $1x10^3$.

	B	Lower 95% CI	Upper 95% CI
Fixed terms			
Intercept	199993.57	-2548.95	51941.85
LP	-70.76	-306.22	123.66
PER	9276.83	-29736.28	43560.62
Body condition	126167.93*	12800.91	237774.08
Male	-2718.92	-12279.24	5966.15
AOX	-14.05	-76.23	42.11
Random terms			
Season	727908374	0.0004	2.321000000
Year	9005370	0.0004	15416149
Residual variance	382089869	1543	768652109

Table 4.3 Autumn survival model variables.

Coefficient estimates (β) are presented along with their 95% confidence intervals (CI). Asterisks denote variables that differed statistically from zero (based on 95% CIs). Note $5x10^6$ iterations, with $1x10^3$ burn-in and thinning interval of $1x10^3$.

	B	Lower 95% CI	Upper 95% CI
Fixed terms			
Intercept	3770	-8028	17250
LP	-152·	-347.4	3.555
PER	-5426	-37210e+04	21690
Body condition	35400	-25580	123900
Male	-413.0	-7078	5399
AOX	0.5056	-35.27	37.76
Random terms			
Season	90574858	0.0003	390084392
Year	19993974	0.0004	42517359
Residual variance	261636378	28291	479741284

Table 4.4 Survival to 1 year, model variables.

Coefficient estimates (β) are presented along with their 95% confidence intervals (CI). Asterisks denote variables that differed statistically from zero (based on 95% CIs). Note $5x10^6$ iterations, with $1x10^3$ burn-in and thinning interval of $1x10^3$.

	B	Lower 95% CI	Upper 95% CI
Fixed terms			
Intercept	2955.175	-12513.594	21112.121
LP	-245.545*	-568.466	5.953
PER	3509.545	-44043.815	50189.684
Body condition	51855.110	-45892.321	168107.218
Male	1725.593	-13161.401	13973.444
AOX	35.079	-34.989	118.385
Random terms			
Season	24500000	0.0008	116400000
Year	13491051	0.0008	44966947
Residual variance	931126264	110171	246800000

4.4.2 Effects of OS on growth

4.4.2.1 Head-body length and zygomatic arch width

Adult head-body length was predicted by cub body condition (fatter cubs grew longer as adults, Table 4.5). Similarly, adult zygomatic arch width was wider for fatter cubs, especially for males (Table 4.6). No metric of oxidative stress was influential.

Table 4.5 Adult body size, model variables.

Coefficient estimates (β) are presented along with their 95% confidence intervals (CI). Asterisks denote variables that differed statistically from zero (based on 95% CIs). Note $5x10^6$ iterations, with $1x10^3$ burn-in and thinning interval of $1x10^3$.

	B	Lower 95% CI	Upper 95% CI
Fixed terms			
Intercept	678.126**	636.776	713.739
LP	-0.022	-0.535	0.453
PER	38.679	-11.196	89.268
Body condition	258.273**	119.697	400.389
Male	4.174	-9.025	14.699
AOX	-0.067	-0.139	0.003
Random terms			
Season	73.92	0.0003733	448.2
Year	1192	0.003172	4399
Residual variance	637.7	375.9	898.6

Table 4.6 Adult zygomatic arch width, model variables.

Coefficient estimates (β) are presented along with their 95% confidence intervals (CI). Asterisks denote variables that differed statistically from zero (based on 95% CIs). Note $5x10^6$ iterations, with $1x10^3$ burn-in and thinning interval of $1x10^3$.

	B	Lower 95% CI	Upper 95% CI
Fixed terms			
Intercept	83.400172	74.689292	92.181418
LP	-0.009260	-0.077	0.0653
PER	1.968690	-4.650	9.067
Body condition	30.837995*	11.943	48.860
Male	2.715015*	0.886	4.356
AOX	-0.005702	-0.016	0.004
Random terms			
Season	0.9583	0.0001	3.22
Year	109.4	0.352	212.000
Residual variance	11.9	7.823	16.71

4.4.2.2 **Adult foot size (pastern length)**

Foot length was also predicted by cub body condition and sex (males had bigger feet than females) as well as cub antioxidant capacity, where cubs with high antioxidant capacity grew to have smaller feet as adults (Table 4.7). That is, investment in antioxidant capacity appeared to come at the cost of foot (skeletal) growth.

Table 4.7 Adult foot size, model variables.

Coefficient estimates (β) are presented along with their 95% confidence intervals (CI). Asterisks denote variables that differed statistically from zero (based on 95% CIs). Note $5x10^6$ iterations, with $1x10^3$ burn-in and thinning interval of $1x10^3$.

	B	Lower 95% CI	Upper 95% CI
Fixed terms			
Intercept	97.486	94.099	101.606
LP	-0.024	-0.104	0.052
PER	1.592	-5.870	9.257
Body condition	42.601*	23.347	63.878
Male	2.311*	0.455	4.325
AOX	-0.012*	-0.023	-0.001
Random terms			
Season	1.863	0.0002	6.113
Year	13.1	0.0002	33.57
Residual variance	14.36	9.315	20.36

4.4.3 **Comparing oxidative stress measurements across age-classes**

4.4.3.1 **Total antioxidant capacity - AOX**

Only season was a significant predictor of antioxidant (Table 4.8). Antioxidant capacity was highest in the summer, but levels between spring and autumn did not differ significantly (Fig. 4.1).

Table 4.8, Model averaging values for age-class AOX models, including relative importance of variables and 99% confidence intervals.

Sqrt(AOX)	Importance	Category level	Estimate	Lower 99% CI	Upper 99% CI
Intercept			11.48	7.09	15.87
Ageclass	1.00	Prime	-8.97	-18.36	0.42
		Old	-10.23	-20.68	0.02
BCI	1.00		3.51	-18.88	25.90
Season	1.00	Summer	2.49*	0.13	4.84
		Autumn	1.83	-1.59	5.25
Age*BCI	1.00	Prime*BCI	31.02	-4.17	66.22
		Old*BCI	36.72	-1.03	74.46
Sex	0.24	Male	-0.02	-1.04	0.86
Age*Season	1.00	Prime*Summer	-1.91	-4.80	0.98
		Old*Summer	-2.8	-5.74	0.13
		Prime*Autumn	0.30	-3.66	4.25
		Old*Autumn	0.22	-3.71	4.14

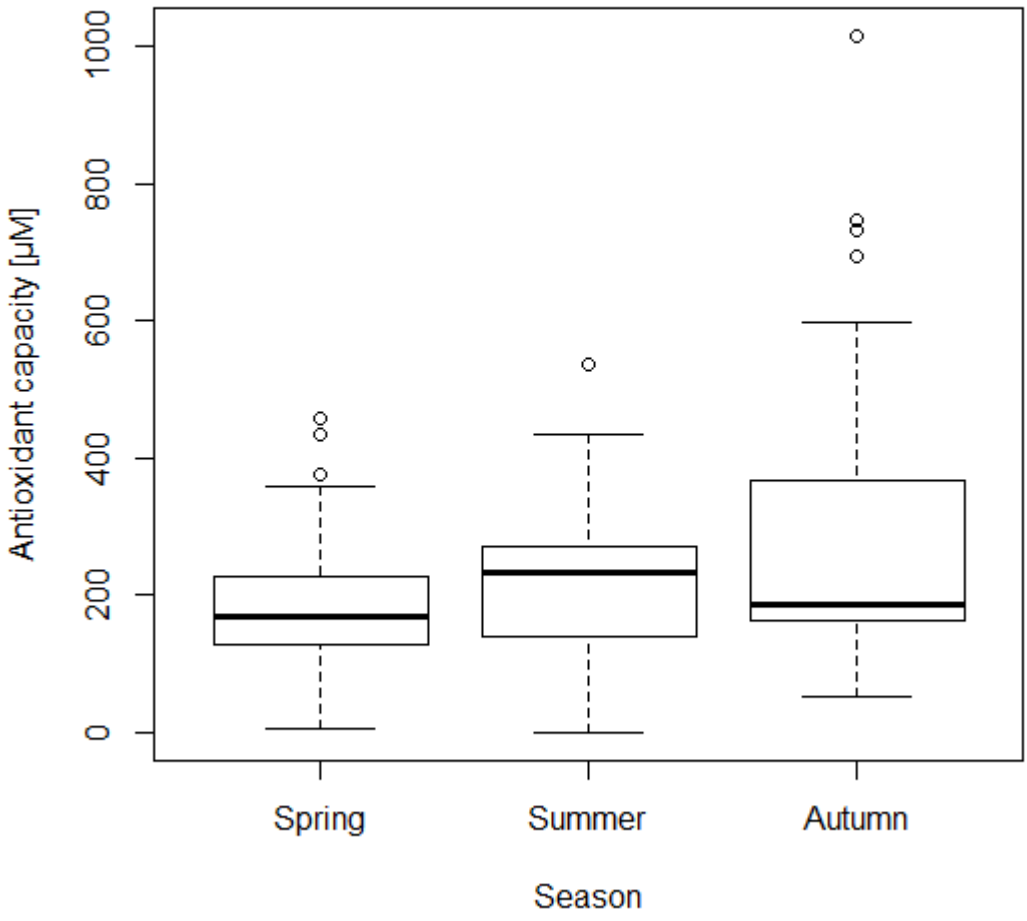


Figure 4.1 Antioxidant capacity by season

4.4.3.2 **Antioxidant enzyme levels - peroxidase**

Again, season was the only significant predictor of peroxidase levels (Table 4.9). Summer levels were significantly higher than in the spring, while autumn levels were significantly lower than spring levels (Fig. 4.2).

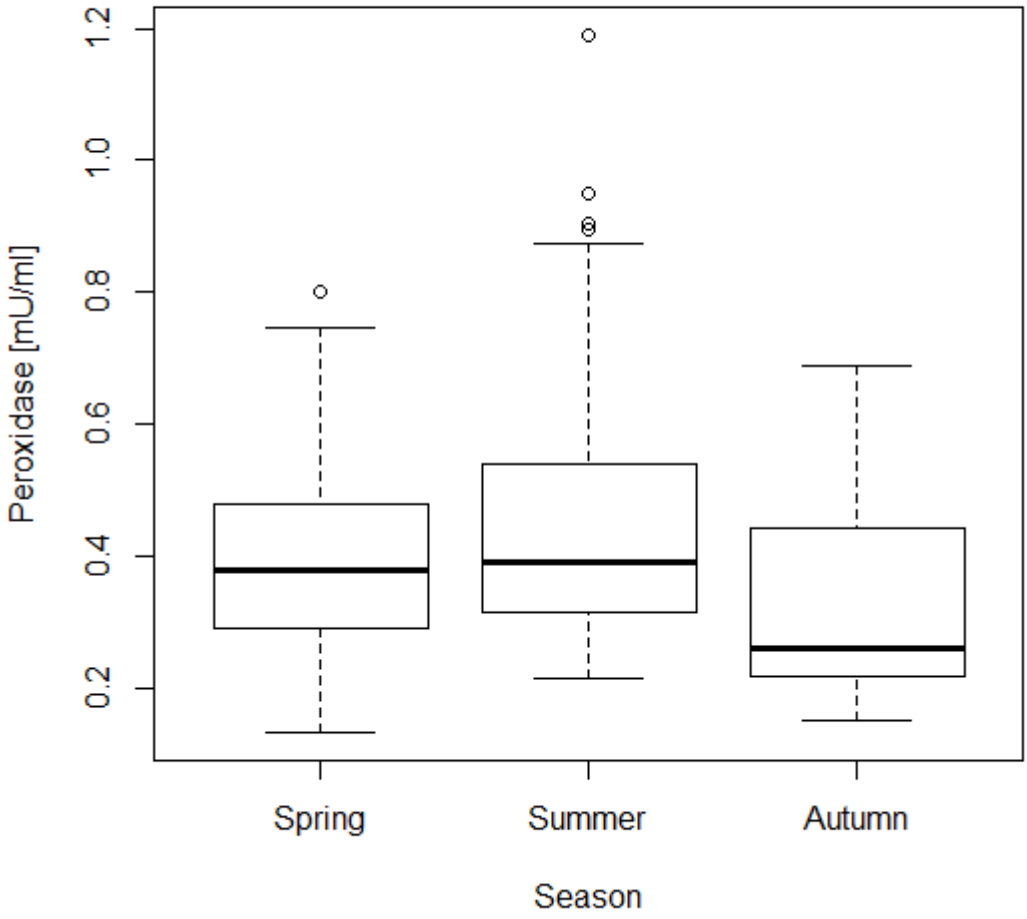


Figure 4.2 Peroxidase levels separated by season.

Table 4.9. Model averaging values for age-class PER models, including relative importance of variables and 99% confidence intervals.

Log(PER)	Importance	Category level	Estimate	Lower 99% CI	Upper 99% CI
Intercept			-1.31	-1.72	-0.90
Ageclass	0.18	Prime	0.01	-0.77	0.90
		Old	-0.10	-1.57	0.39
BCI	1.00		0.93	-0.81	2.68
Season	1.00	Summer*	0.11	0.01	0.22
		Autumn*	-0.31	-0.44	-0.18
Age*BCI	0.18	Prime*BCI	0.06	-2.50	3.25
		Old*BCI	0.45	-0.78	5.90

4.4.3.3 **Oxidative damage - lipid peroxidation**

Oxidative damage was significantly associated with age-class, season, the interaction between BCI and age-class (Table 4.10). Summer levels were significantly lower than spring levels, with autumn levels not differing significantly from spring levels (Fig. 4.3). Overall cubs had higher levels of oxidative damage than did adults of any age, with the caveat that fatter prime aged adults had higher levels of lipid peroxidation than thinner ones (Fig. 4.4).

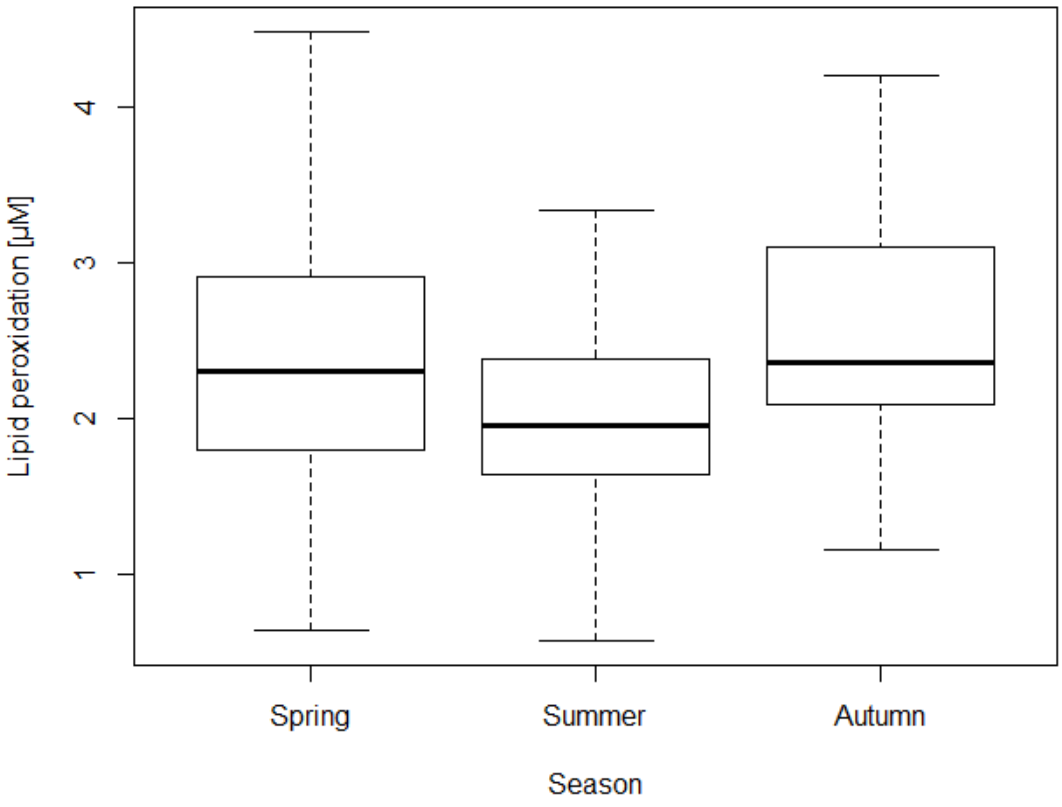


Figure 4.3 Lipid peroxidation by season.

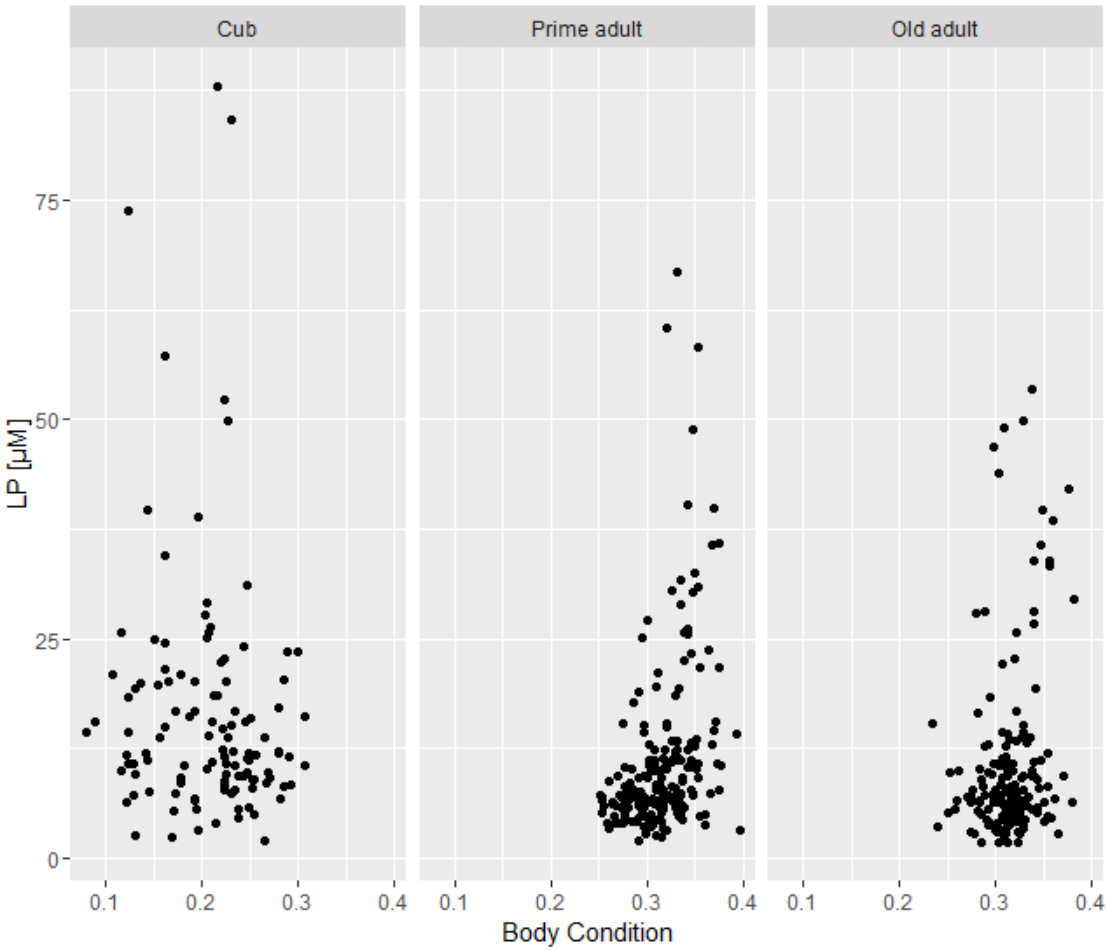


Figure 4.4 Lipid peroxidation levels separated by age-class and body condition.

Table 4.10. Model averaging values for age-class LP models, including relative importance of variables and 99% confidence intervals.

Log(LP)	Importance	Category level	Estimate	Lower 99% CI	Upper 99% CI
Intercept			3.09	2.12	4.06
Ageclass	1.00	Prime*	-2.39	-3.66	-1.11
		Old*	-1.82	-3.30	-0.33
BCI	1.00		-1.75	-5.03	1.53
Season	1.00	Summer*	-0.37	-0.66	-0.07
		Autumn	0.37	-0.12	0.86
Age*BCI	1.00	Prime*BCI*	6.94	2.06	11.83
		Old*BCI	4.90	-0.53	10.33
Sex	0.11	Male	-0.01	-0.08	0.06
Age*Season	0.11	Prime*Summer	0.05	-0.32	0.42
		Old*Summer	0.03	-0.24	0.31
		Prime*Autumn	0.07	-0.48	0.62
		Old*Autumn	0.07	-0.46	0.59

4.4.3.4 **Resistance to oxidative stress – RBC ½-life**

The only significant association with RBC *½-life* length was with season (Table 4.11). RBC *½-life* was significantly lower in summer than in spring and significantly higher in the autumn (Fig. 4.5).

Table 4.11 Model averaging values for age-class RBC ½-life models, including relative importance of variables and 99% confidence intervals.

Log(RBC)	Importance	Category	Estimate	Lower 99% CI	Upper 99% CI
Intercept			4.20	4.07	4.34
BCI	0.36		0.07	-0.21	0.35
Season	1.00	Summer*	-0.06	-0.09	-0.03
		Autumn*	0.08	0.0391	0.12

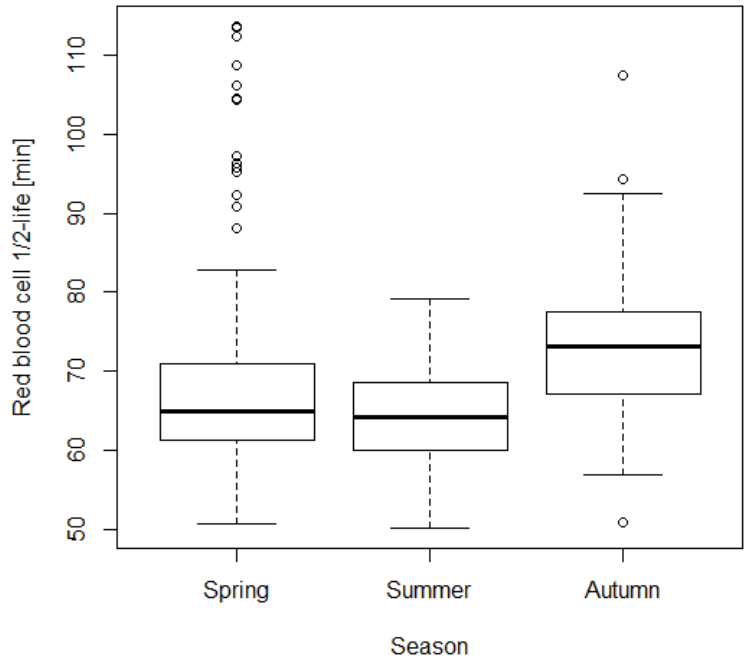


Figure 4.5 Red blood cell ½-life by season.

4.4.4 **Body condition**

Cub BCI increased throughout their first year of development (Fig. 4.6, spring mean = 0.204, SD= 0.052, n= 88; summer mean=0.234, SD=0.034, n=42; autumn =0.261, SD=0.031, n= 16, p<0.001). Mean BCI for prime-aged adults was not statistically different between spring and summer (spring mean = 0.306, SD= 0.023, n=79; summer mean=0.307, SD=0.030; n=92; p=062), but was significantly higher in the autumn (autumn =0.341, SD=0.027, n= 66, p<0.001). In old adults, spring and summer BCI did not differ significantly (spring mean = 0.312, SD= 0.023, n=70; summer mean=0.309, SD=0.024, n=74; p=0.51) but were significantly higher in the autumn (autumn =0.329, SD=0.028, n=48; p<0.001).

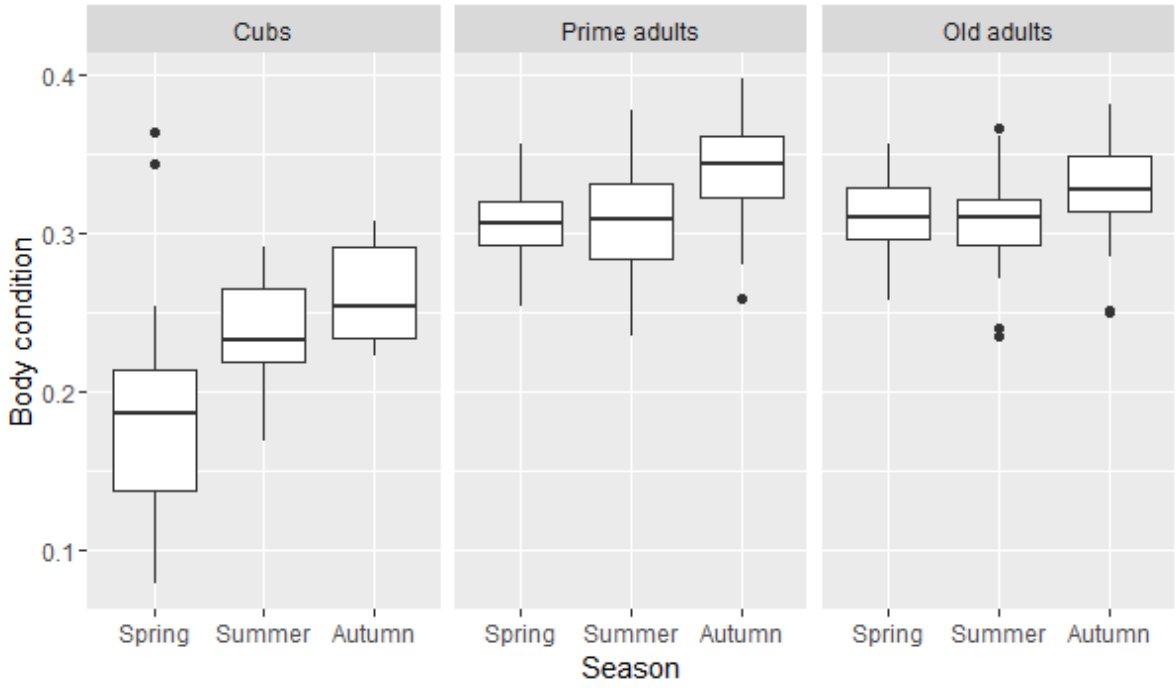


Figure 4.6 BCI for age-classes between seasons

4.4.5 **Reproduction and lactation**

For females two years or older, and thus capable of reproducing, I found no difference in antioxidant capacity, peroxidase levels or RBC ½ -life between those females that

had lactated and those that had not lactated. Oxidative damage (LP), however, was significantly lower for females that had not lactated (mean=10.14662, SE=1.378, n=43) versus females that had (mean=20.525, SE=1.778, n=43; p-value=0.024 on log transformed data), implying that lactation was associated with oxidative damage (Fig. 4.7).

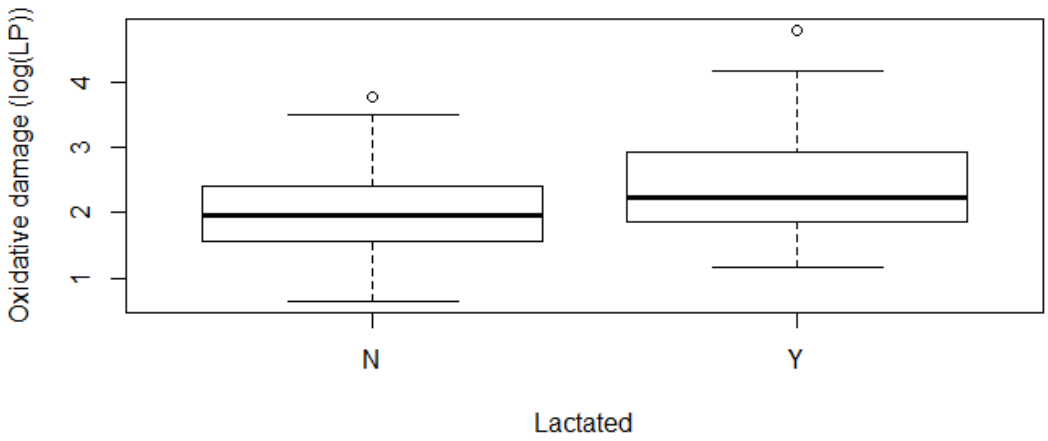


Figure 4.7 Oxidative damage between females that had and had not lactated (n=43 for both categories).

4.4.6 Longitudinal analysis of ageing on oxidative stress biomarkers

4.4.6.1 Tracking within-individual changes in antioxidant capacity with advancing age in adult badgers

There was no indication that AOX, PER, LP or RBC ½-life changed as individuals aged, nor was this sex-dependent. The null model was the only one retained in the ΔAIC cut off for PER, LP and AOX, while sex was retained at low importance for AOX (Table 4.12).

Table 4.12. Model averaging values for longitudinal ageing AOX models, including relative importance of variables and 95% confidence intervals.

AOX	Importance	Category level	θ	Lower 99% CI	Upper 99% CI
Intercept		-	14.28	13.00	15.56
Sex	0.29	Female Male	- 0.01	- -0.61	- 0.63

In summary, cub survival was mainly predicted by body condition in their first few months, followed by a marginal link of oxidative damage levels on survival through their first year. Adult size was linked to cub body condition and sex, with an indication of trade-offs between antioxidant capacity and foot size.

PER and RBC ½-life were only linked to seasonal variations, whereas lipid peroxidation and antioxidant capacity were also linked to age-class and body condition. Females that had lactated had higher levels of lipid peroxidation than females that had not.

Finally, there was no suggestion that ageing of individual badger lead to increased oxidative damage or decrease antioxidant capacity.

4.5 Discussion

4.5.1 Impacts of OS on juvenile development and survival

Despite the small sample size, oxidative stress in cubs occurring between August (summer) and November was a strong predictor of survival through the ensuing winter to age one (maturity). Oxidative damage to lipids, DNA and polysaccharides can lead to premature cell death (apoptosis) and is worsened by rapid growth rates in mammals (Nussey *et al.* 2009). In juveniles, trade-offs in resources are common, where cubs have limited resources that can be invested in somatic growth or other traits such as immunity (Soler *et al.* 2003, van der Most *et al.* 2011) and resistance to oxidative stress

(Dowling and Simmons 2009, Monaghan *et al.* 2009, Metcalfe and Alonso-Alvarez 2010). In spite of lipid peroxidation being an important factor in cub survival probability, there were no effects of this damage to the ultimately achieved adult body-length. Adult body-length was only predicted by cub BCI as was zygomatic arch width, but with a sex effect (male heads were broader than females'); this suggests that compensatory growth does not occur among cubs that suffer resource restriction early in life (Feder *et al.* 2008). This is consistent with patterns reported for other mammals, where restricted early growth leads to smaller adults and lower lifetime reproductive success (Gaillard *et al.* 2003, Parker *et al.* 2009).

Interestingly, foot size was linked not only to body condition and sex, but also to antioxidant capacity as cubs, where high investment in AOX by cubs was traded-off against smaller adult foot size (see Monaghan 2008, Kim *et al.* 2011).

In summary, here, I see a pattern where 'fatter' cubs consistently grow to larger adult sizes, across all morphometrics, but with a partial signal for investment in AOX compromising this relationship. I re-emphasise here that developmental traits exhibited by cubs can be measured only among survivors, where on average across our three study years 60% of cubs died. Reasonably, fatter cubs are more likely to survive in the first instance, potentially muffling any signal evidencing a trade-off between antioxidant capacity and adult body-size attained. Ability to detect trade-offs is thus clouded by the fact that the 'harsh' conditions predicted to produce trade-offs, can readily cause individuals to be totally overwhelmed, and ultimately to die. In-so-far that a trade-off signal was only detected in adult foot size, it is notable that feet grow rapidly in young carnivores (e.g. the big paws of puppies and kittens), and are thus further

along the allometric trajectory toward full adult size by the time I first measured them. This could imply that up until first capture (weaning and independence in May / June) an antioxidant capacity able to cope with OS (notably arising from neonatal coccidial infections; Newman *et al.* 2001) is critical to early survival because BCI is taken care of by maternal lactation. After this point, successful independent feeding, to maintain BCI, becomes more critical.

It is also important to note that juveniles are generally more susceptible to disease and parasites which can affect ROS production as well as body condition, size and survival (Newman *et al.* 2012). These factors were not taken into account in the analysis but could be confounding factors.

With regard to phenotypes, selective yearly mortality (through OD) and the small number of cubs (44) that survived to adulthood over the three years possibly obscured subsequent physiological effects. This might have limited our ability to detect divergent adaptive strategies best fitted to different selection pressures (Baquedano *et al.* 2008). Nevertheless, I also found no responses that contradicted the potential for cub phenotypes, as there was substantially more variation in years where cubs of different quality were able to survive to the first trapping session in May. In line with Cohen and McGraw (2009; looking at birds), I would need many more years of data, spanning substantial inter-annual variation in conditions driving cohort mortality rate to distinguish phenotypes from evolutionary lability and/or ecological heterogeneity.

4.5.2 **Life-history interactions with OS**

A preliminary study in a harsh year where very few cubs survived (2011) showed that cubs had higher antioxidant capacity than older individuals (Bilham *et al.* 2013). In this present study, however, there was no indication that cubs had higher levels of antioxidant capacity. Rather that antioxidant capacity only varied between seasons,

where it was highest in the summer, but did not differ between spring and autumn. These high levels of antioxidant capacity happened when antioxidant capacity was lowest, suggesting an upregulation of antioxidant defences in order to quench ROS, and avoid oxidative damage (Halliwell & Gutteridge 2015). Lipid peroxidation also varied between age classes, with cubs showing higher levels of OD than did prime and old adults. In badger cubs, the potential for OS is high due to metabolic demands of growth (increased ROS leakage through the electron transport chain); therefore, having low antioxidant capacity and 'average' peroxidase levels might not be enough to avoid oxidative damage. Birth is a major oxidative challenge for cubs, followed by substantial early energetic demands to support growth and development, leading to increased ROS production (Gaál *et al.* 2006). During suckling antioxidant rich colostrum is able to help mitigate much of this neonatal oxidative stress (Przybylska *et al.* 2007). Weaning can, however, cause a spike in OD, as has been reported in dairy calves, attributed to rapid growth spurts and the transition from milk to adult food sources (Gaál *et al.* 2006). Indeed, on average, young cubs exhibited significantly higher OD (lipid peroxidation), than did adults in the spring. As cubs start developing adaptive immunity to coccidial infection in the late summer of their first year (Newman *et al.* 2001) this will decrease reliance on innate immunity and antioxidant defences, and thus reduce the negative effects of ROS (Kohchi *et al.* 2009); traits likely to be related to this drop in lipid peroxidation. Nussey *et al.* (2009) found similar results in soay sheep, where lambs had significantly higher lipid peroxidation levels than did adults of any age-class.

The fact that prime-aged adults have higher OS when they are fatter either suggests that the higher levels of foraging required to increase their body mass is heightening ROS production and therefore OD (Liu *et al.* 2000). There is a similar trend in old adults, although this is not statistically significant. It should also be noted that high circulating

levels of poly unsaturated fatty acids could be interfering with the TBARS assay and that higher fat mass could be leading to this (Keany *et al.* 2003).

Peroxidase was was not significantly explained by age-class, counter to Hazelton and Lang' (1985) finding that antioxidant enzymes decreased with age in laboratory mice, but rather followed the same trends as AOX, where summer levels were the highest in all age classes. I also found no indication that juveniles show low levels of antioxidant enzymes (Barata *et al.* 2005); although the late capture of cubs (when they were already weaned), is potentially obscuring the initial development of antioxidant enzyme defences of new-born badgers (Khan and Black 2003). Glutathione peroxidase is an important enzyme in maintaining the redox balance in the body, and is involved in the oxido-reduction of glutathione, and the neutralisation of peroxides as well as in transforming lipid peroxides into alcohols (Abraham *et al.* 1978); where deterioration in glutathione production in humans leads to grey hair, due to pigment oxidation (Trueb 2009). It is important for organisms to be able to terminate lipid peroxidation chain reactions, else these self-propagate to exacerbate damage (Mylonas and Kouretas 1998). In badgers, across all age-classes resilience to OS was lower in the summer, despite higher antioxidant capacity.. Because RBC ½-life can indicate damage which has occurred over the past 110 days (the time that RBC stay in circulation), it is possible that these damages are reflecting the harsh conditions experienced over the summer, where food and water shortages can lead to significant physiological distress (Nouvellet *et al.* 2013). Indeed, RBC ½-life was longest in the autumn, where food availability is at its highest.

4.5.3 **Effects of reproduction and lactation on OS**

No differences in female levels of antioxidant defences (both AOX and peroxidase) nor in the resistance to oxidative stress (RBC ½-life) were apparent during the spring cub-rearing season in our study; however, there was a significant difference in lipid peroxidation levels between females that had lactated and those that had not. This likely arises from increased energetic demands during pregnancy and lactation (Castillo *et al.* 2005, Crocker *et al.* 2016). It is not necessarily surprising that I did not find any differences in the other measurements of oxidative stress when taking data from an un-manipulated population. Not least, almost all sexually mature females mate, whereas only ca. 30% bear offspring (reviewed in Macdonald *et al.* 2015a), and so those not conceiving or implanting embryos, or aborting embryos likely do so because they experience different, but greater, stress than successful mothers. This could be a longevity assurance mechanism, where it might be more beneficial for LRS to invest in somatic maintenance that year (Edward and Chapman 2011). Metcalfe and Monaghan (2013) suggest that manipulation of reproductive success might be needed in order to investigate the effects of reproduction on oxidative stress. Moreover, a recent meta-analysis by (Blount *et al.* 2015) highlighted the difficulties in measuring OS effects of pregnancy and lactation and found that in many studies having only two groups (has and has not reproduced) was not sufficient to observe consistent differences in lipid peroxidation levels in blood samples, despite strong effects on protein damage (see also Fletcher *et al.* 2013).

4.5.4 **Longitudinal analysis of ageing on oxidative stress biomarkers**

With respect to the FRTA (Beckman and Ames 1998), where OS levels are expected to increase, and antioxidant defences decrease as animals age, and using age as a continuous variable within individuals, I found no significant difference in oxidative

damage, molecular or enzymatic defences nor any change in resilience to oxidative stress with advancing age. Despite finding no changes in plasma concentrations of lipid peroxidation with age among adults, this does not necessarily infer that organ tissue levels mirror this pattern (Haider *et al.* 2014). This is a limitation to non-lethal ecological studies, because plasma data do not necessarily reflect organ tissue levels, which could still be at higher risk of lipid peroxidation with advancing age, as suggested by the FRTA. Another hypothesis is that in order to maximise lifetime reproductive success (LRS), longevity assurance mechanisms ensure that ageing individuals invest sufficiently in somatic maintenance in order to survive until the next breeding season in adequate reproductive condition (Edward and Chapman 2011).

4.6 **Conclusion**

Oxidative damage is higher in badger cubs, suggesting that the high demands of development (such as high metabolic rate) coupled with the higher likelihood of juveniles being affected by disease increases the risk of oxidative damage. I also found a trend for cubs with higher lipid peroxidation levels to be less likely to survive their first year thus opening up avenues of study for conservation practitioners, where juvenile survival is often an important factor in the success of reintroduction programs and conservation of endangered species. I thus urge conservation scientists to investigate the factors resulting in high levels of oxidative damage in juveniles (see Beaulieu *et al.* 2013) as well as the potential trade-offs which occur with investment in antioxidant defences during development (Monaghan 2008, Monaghan *et al.* 2009). Despite female reproduction having a physiological cost, I did not find any data suggesting that these damages accumulate and increase levels of OD with ageing (Finkel and Holbrook 2000). In this K-selected species, longevity assurance mechanisms might be modulating reproduction in order to maximise survival and LRS.

Chapter 5: **Oxidative stress: how weather conditions can impact individuals, modelled using the European badger.**

5.1 **Abstract**

Underlying wild-animal demographic responses to weather variability, inclement conditions stress individuals at the physiological level, generating reactive oxygen species causing oxidative stress and tissue damage. Our three-year field study investigated the effects of seasonal weather conditions on biomarkers for oxidative stress, oxidative damage and antioxidant defence in the European badger (*Meles meles*). We found age-class effects: cubs were more susceptible to oxidative stress and oxidative damage than adults, especially very young cubs in the spring, when they also exhibited lower AOX biomarkers than adults. Oxidative stress responses were incongruent with previous badger survival analyses. We found greater oxidative damage among cubs experiencing intermediate spring and summer rainfall and warmer temperatures; conditions previously linked to greater survival probability. Oxidative damage was high in cubs even when antioxidant biomarkers remained high. Adult responses paralleled previous survival analyses, with wetter spring and summer conditions associated with higher oxidative damage but also higher antioxidant biomarkers. Autumnal weather did not vary substantially from normative long-term trends during our study and thus weather effects were muted. Winter carry over effects were partially evident, with drier and milder conditions associated with greater oxidative damage in the following spring, but also with higher antioxidant capacity – plausibly warmer conditions promoted more badger activity , with associated metabolic costs at a time of year when food supply is limited. This type of integrative and interdisciplinary understanding of the mechanisms underscoring adaptive capabilities

provides insight into how future climate change may impact wild populations, facilitating the design of appropriate conservation strategies.

5.2 **Introduction**

When animals experience low food availability or disease, increased metabolic stress (e.g. due to extended foraging forays, immune responses, etc) promotes the generation reactive oxygen species (ROS) (Leeuwenburgh & Heinecke 2001). Although animals produce endogenous antioxidant molecules (e.g. glutathione), or enzymes (e.g. peroxidases; supplemented by their consumption of exogenous dietary antioxidants) to quench ROS (by neutralising the unpaired electron), oxidative stress (OS) and oxidative damage (OD) can arise if antioxidant defences are exceeded (Matés *et al.* 2002). Consequently, when evolutionarily novel stressors arise from Human Induced Rapid Environmental Change (HIREC; Sih 2013) these can further exacerbate the physiological burden wild-living animals must cope with (Sies 1997), placing their defence systems under severe, and potentially unsustainable strain.

Climate change is a principal HIREC factor (e.g., Parmesan 2006), and weather conditions are often intrinsically linked to food availability, foraging success, activity patterns, and thermoregulatory costs (Kronfeld-Schor & Dayan 2013). Therefore, increasingly unseasonable, variable and extreme weather (IPCC, 2014; see also Parmesan, Root & Willig 2000) is likely to exceed the ability of species to tolerate changed conditions (Smith *et al*. 2000), with implications for fitness and survival (White 2008). Furthermore, there can be cumulative 'carry-over effects' (COE; Harrison *et al.* 2011), where weather conditions in one season affect an individual's subsequent performance, provided it continues to survive. Ultimately, therefore,

differences in the relative effects of both proximate and cumulative weather conditions between individuals can drive natural selection (Parmesan *et al.* 2000).

Although many studies of terrestrial mammals have examined weather-induced macro-demographic responses to climate change (see Newman & Macdonald 2015), as-well-as individual declines in body-condition and reproductive success (e.g. beavers, *Castor fiber L.*: Campbell *et al.* 2012; red deer, *Cervus elaphus L.*: Moyes *et al.* 2011; badgers: Byrne *et al.* 2015; Soay sheep, *Ovies aries L.*: Coulson *et al.* 2001), observing the phenomenology of weather interactions with species ecology does not reveal the causal mechanisms involved (McGraw *et al.* 2010; Metcalfe & Alonso-Alvarez 2010). Oxidative stress is increasingly recognised as one of the major factors influencing life-history traits in ecology (e.g., Monaghan *et al.* 2009) ; however, it remains a priority to better understand how OS operates mechanistically as the currency (*sensu*, Metcalfe & Alonso-Alvarez 2010) through which weather and climate can affect physiology. In comparison, a number of studies have found an interaction between oxidative stress and water temperature in marine environments (reviewed by Lesser 2006; see also Pörtner 2001), and acute heat stress is a recognised animal health issue when rearing poultry (e.g., Lin *et al.* 2006)

A variety of effective biomarkers of chronic oxidative stress responses have been established. For instance, OD can be detected as lipid peroxidation (Mylonas & Kouretas 1998), via plasma malondialdehyde (MDA; Nielsen *et al*. 1997), which is a biomarker of long-term tissue damage and is associated with various diseases.

Similarly resistance to OS can be measured via the ability of red blood cells (RBCs) to survive a free radical attack *ex vivo* (Bize *et al.* 2008). RBCs survive for around 120 days in the bloodstream and so provide a good indicator of OS over this timeframe (Kurata *et al.* 1993). Antioxidant defences are generally measured as total non-

enzymatic antioxidant capacity, and/ or by enzyme activity levels (see Somogyi *et al.* 2007), with higher responses conferring greater protection against oxidative stress. Since antioxidant defences are costly to develop and maintain, they may be traded-off against investment in growth and reproduction (Alonso-Alvarez *et al.* 2007b, Horak *et al.* 2007). Crucially, the full redox balance system should be investigated, where many studies have focused solely on antioxidant defences, erroneously assuming that this will indicate levels of OS. Absolute antioxidant levels are, in fact, only informative if the levels of ROS or OD are also known (Monaghan *et al.* 2009).

Here we present a novel inter-disciplinary study, examining the interaction between seasonal weather conditions, OS, OD, antioxidant capacity (AOX) and enzymatic antioxidant capacity (peroxidise, PER) for a medium-sized generalist carnivore, the European badger (henceforth 'badger');. Badger ecology has proven intimately linked to weather conditions, in terms of trans-continental biogeographical distribution (Johnson *et al.* 2002; Silva *et al.* in press), survival dynamics (Macdonald & Newman 2002, Macdonald *et al.* 2010) life-history factors (Nouvellet *et al.* 2013), body condition (Byrne *et al.* 2015), and activity patterns (Noonan *et al.* 2014; 2015). Badgers utilise fossorial communal burrows (termed setts) and can stay underground, protected from inclement weather, if they have sufficient body-fat reserves to do so (see Noonan *et al.* 2014). In winter,when their preferred earthworm (*Lumbricus sp*.) food source is less available (Curry 2004), badgers also use torpor to mitigate food scarcity (Newman *et al.* 2011). Ultimately, however, when badgers do not meet their immediate calorific needs they catabolise fat reserves (Kunz *et al.* 1998). Fat catabolism generates ROS (Morales *et al.* 2004) and causes redox imbalance, leading to increased levels of OD, despite upregulation of antioxidant enzymes (Morales *et al.* 2004, Vijayakumar *et al.* 2004). Once fat reserves are depleted, catabolising muscle (protein) can further increase

OS (Eisler *et al.* 2004; Finn and Dice 2006). Certainly, in badgers, body-fat depletion adversely affects health (Domingo-Roura *et al.* 2001), over-winter survival (Macdonald and Newman 2002) and embryonic implantation (Woodroffe 1995; Macdonald *et al.* 2015). Badgers therefore seek to avoid net energy loss via managing their activity regimes and their extent of above-ground emergence (Noonan *et al.* 2014; 2015). Crucially, provided food is readily available, high foraging costs will be sustainable, albeit with high metabolic turn-over, which generates ROS (Leeuwenburgh & Heinecke 2001). Additionally, badgers also experience short-term periodic swings in foraging activity and foraging success, allowing them to cyclically replenish depleted somatic reserves. Consequently, OS / OD could occur even without apparent loss of body-condition over a season, due to the effective compensation of reserves.

Previous work in this same badger population by Macdonald and Newman (2002) identified that spring rainfall was critical to cub survival, with drought conditions leading to higher mortality rates. Building on this, Nouvellet *et al.* (2013) found distinct age-class specific responses, where cub survival probability was actually highest in years with intermediate annual rainfall (neither too wet nor too dry; a negative quadratic relationship) and intermediate temperature; whereas adult survival probability was greater in wetter years. We therefore posit that, if weather interactions with OS/ OD biomarkers follow similar patterns, cub cohorts will experience more severe OS effects in years with less moderate weather, whereas adults will benefit from more rainfall. We then explore if weather conditions relate to inter-individual variability in OS / OD biomarkers, possibly favouring different OS phenotypes (*sensu* Metcalfe & Alonso-Alvarez 2010).

Winter weather is especially critical for badgers, with frost making earthworms unavailable. This has been linked with lower over-winter survival rates (Macdonald &

Newman 2002), as-well-as lower cub recruitment into the adult population (Nouvellet *et al*. 2013). This leads us to further hypothesise that winter weather COE may affect adult OS and antioxidant levels in the following spring, as an indicator of individual quality.

Short-term observational studies of weather effects are always hostage to fortune, because substantial variation may not occur within the study period (Weins 1977); nevertheless our study years included sufficient weather deviation from long-term normative values to show meaningful effects on biomarkers (Table 5.1). Crucially, our study included winters with substantially different numbers of frost days, where it is not absolute winter temperature that seems important to badger ecology, but rather the tipping point between frost versus no frost, affecting earthworm availability (Macdonald *et al.* 2010).

This intricate approach to understanding weather effects is important to expand the growing field of eco-physiology, which aims to establish the cellular mechanisms connected to population dynamics (Costantini *et al.* 2010). Furthermore, it provides a new and ingenious tool for anticipating the effects of climate change on wildlife, enabling appropriate and effective conservation action (Beaulieu *et al*. 2013). Indeed, Berteaux *et al.* (2006) identify a lack of understanding on proximate causality as one of the main constraints when projecting the effects of climate change on mammals.

In this chapter I specifically, I test for

- COE of winter weather conditions on adult OS levels in the spring, using OS physiology as a marker of individual quality in the spring.
- Effects of weather conditions in the 3 months preceding sampling on OS measurements. Here I hypothesise that seasonal weather will have specific effects on antioxidant capacity and oxidative stress, in relation

to badger annual life-history routines (e.g., reproduction, fattening for winter, winter catabolism etc).

- Differential effects of weather conditions on cub antioxidant development and OS levels. I investigate whether more severe effects of OS are observed on cub cohorts in years with more extreme weather, potentially corresponding to different phenotypes (see Metcalfe and Alonso-Alvarez 2010).
- Weather from the day before (temperature and rain) will be added into the seasonal models in order to confirm that the measurements are not mainly picking up short term variation.

5.3 Methods

5.3.1 Trapping & sampling

Badgers were caught as part of an ongoing socio-ecological study in Wytham Woods, UK (Macdonald *et al.* 2015a, see Chapter 2 for further details). Briefly, marked individuals were captured seasonally (spring (2012-14), summer (2012-14), autumn (2012-13), sedated, and blood samples were taken. Individuals were given time to sufficient time to recover from sedation before being released back at their sett (Sun *et al.* 2015). A body condition index (BCI) was calculated from body-length and weight measurements as: $\log_e$(weight)/$\log_e$(length), (Macdonald and Newman 2002, Noonan *et al.* 2014).

5.3.2 Oxidative stress assays

Total antioxidant capacity (AOX), peroxidase (PER), lipid peroxidation (LP) and red blood cell ½-life (RBC ½-life), Assays were run for a total of 568 blood samples (146

cubs, 235 prime adults and 187 old adults) from 280 individuals, of which 102 were cubs.. For details of the assays and how they were performed, see Chapter 2.

5.3.3 **Weather data**

Weather records were obtained from the University of Oxford's Radcliffe Meteorological Station, which are freely available (http://www.geog.ox.ac.uk/research/climate /rms/reports.html). Metrics of rainfall (total mm/month), minimum temperature (monthly average of daily minimum temperatures, in degrees C), maximum temperature (monthly average of daily maximum temperatures, in degrees C) and frost (number of days of frost/month) were extracted from this dataset and average values were calculated per season, defined as: **Winter**: December, January, February; **Spring** (preceding spring trapping session): March, April, May; **Summer** (preceding summer trapping session): June, July, August; **Autumn** (preceding autumn trapping session): September, October, November. Badgers were not trapped in winter due to welfare concerns linked to risk stressing pregnant females, and due to low trap success (see Macdonald *et al.* 2015a). Comparisons of how these seasons compared to 30 year average conditions for these same periods are presented in Table 5.1. Mean daily temperature and rainfall measurements were also calculated for the day prior to each trapping / measurement.

Table 5.1 Weather conditions in Oxford for the three study years. Averages for the last 30 years are also presented.

Rainfall (mm/month)	**30 year average**	**2012**	**2013**	**2014**		**Max temp (°C)**	**30 year average**	**2012**	**2013**	**2014**
Winter	55.78	42.37	69.73	111.57		Winter	7.85	8.8	7.07	9.67
Spring	51.26	74.27	55.87	59.9		Spring	13.94	14.77	11.70	14.97
Summer	52.25	112.43	27.43	56.00		Summer	21.72	20.40	22.63	22.4
Autumn	62.88	88.36	60.77	NA		Autumn	14.88	13.97	15.07	NA
Min temp (°C)	**30 year average**	**2012**	**2013**	**2014**		**Winter frost (total days)**	**30 year average**	**2012**	**2013**	**2014**
Winter	2.18	2.63	1.80	3.77			26	26	50	5
Spring	5.57	5.97	3.60	6.27						
Summer	12.25	12.60	12.40	12.33						
Autumn	7.75	6.83	7.87	NA						

5.3.4 PCAs for weather data

In order to investigate COE of winter conditions on OS metrics and investigate effects of seasonal weather conditions on different age-classes, weather metrics had to be pre-analysed to avoid inter-correlation. A principal component analysis (PCA) was thus applied across these continuous data to generate independent variables. This procedure resulted in the retention of two principal components (PC) as predictive variables (determined using the R function prcomp; Crawley 2002), which cumulatively summarised 100% of the variance in the weather data for the winter weather analysis, 99.6% for the seasonal age-class analysis (see Tables 5.2 & 5.3).

5.3.4.1 PCA for winter weather COE

Factor loadings indicated that temperature (maximum temperature, minimum temperature and number of days of frost over the winter) were the most influential parameters along the PC1 axis; temperatures with a negative loading, frost positive (see Table 5.2). That is, higher values of PC1 correspond to lower temperatures and more

frost. PC2 was determined primarily by a negative loading of rainfall, where higher values of PC2 correspond to drier weather. For clarity, components are henceforth referred to as PCtemp and PCrain respectively.

Table 5.2 Summary of the first two principal components of winter weather data

Variable	PC1	PC2
Eigenvalue	3.25	0.75
% of variance explained	81.24	18.76
Cumulative % of variance explained	81.24	100
Days of frost	0.549	-0.184
Minimum temperature	-0.527	0.364
Maximum temperature	-0.556	0.025
Rainfall	-0.335	-0.913

5.3.4.2 **PCA seasonal analyses**

Factor loadings indicate that temperature (maximum temperature and minimum temperature) was the most influential parameters along the PC1 axis, with negative loadings; thus higher values of PC1 correspond to lower temperatures (see Table 5.3). PC2 was determined a positive loading of rainfall, where higher PC2 values correspond to wetter conditions. Again, these components are henceforth referred to as PCtemp and PCrain

Table 5.3 Summary of the first two principal components for the seasonal weather analysis.

Variable	PC1	PC2
Eigenvalue	1.972	1.016
% of variance explained	65.730	33.870
Cumulative % of variance explained	65.730	99.600
Minimum temperature	-0.703	0.135
Maximum temperature	-0.709	-0.059
Rainfall	0.054	0.989

5.3.5 Statistical analysis

5.3.5.1 Analysis of winter weather COE on adult OS and antioxidant defences in the following spring

Utilising these principal weather components, along with BCI and sex, models were built to examine interactions with each OS measurement (antioxidant capacity – AOX, lipid peroxidation – LP, peroxidase – PER and red blood cell ½ life – RBC ½-life). Badger ID and age-class were included as random effects in these models to account for repeat sampling, and for any differences between age-classes (see Chapter 4). For each model, AICc was derived (AIC adjusted for small sample size in relation to the number of parameters, where a smaller value indicates stronger support for the model) and the relative weight of each model (W).

AOX = f (PCrain+ PCtemp +Sex+BCI) + (1|Social Group/ BadgerID)

Log(LP) = f (PCrain+ PCtemp +Sex+BCI) + (1|Social Group/ BadgerID)

PER = f (PCrain+ PCtemp +Sex+BCI) + (1|Social Group/ BadgerID)

RBC ½-life = f (PCrain+ PCtemp +Sex+BCI) + (1|Social Group/ BadgerID)

5.3.5.2 Analysis of seasonal weather effects on cub and adult OS & antioxidant defences

Using these principal components, along with BCI, age-class (cub, prime adult, old adult, see Chapter 4), sex and the interactions between each PC, season and age-class, models were built to examine prediction of each OS measurement. The models below were run with and without interactions, where badger ID was included as a random effect to account for repeat sampling of individuals.

SQRT(AOX) = *f* (Age-class * PCrain + Season * PCrain + Age-class * PCtemp + PCtemp * Season + Ageclass * daytemp + Ageclass * dayrain + Sex + BCI) + (1|Social Group/ BadgerID)

Log(LP) = *f* (Age-class * PCrain + Season * PCrain + Age-class * PCtemp + PCtemp * Season + Ageclass * daytemp + Ageclass * dayrain + Sex + BCI) + (1|Social Group/ BadgerID)

Log(PER) = *f* (Age-class * PCrain + Season * PCrain + Age-class * PCtemp + PCtemp * Season + Ageclass * daytemp + Ageclass * dayrain + Sex + BCI) + (1|Social Group/ BadgerID)Log(RBC ½-life) = *f* (Age-class * PCrain + Season * PCrain + Age-class * PCtemp + PCtemp * Season + Ageclass * daytemp + Ageclass * dayrain + Sex + BCI) + (1|Social Group/ BadgerID)

AICc was derived for each model along with relative weight (W).

5.3.5.3 **Model averaging**

Model averaging (Symonds and Moussalli 2011) was applied in all analyses to models for ΔAIC<4, with associated 99% confidence intervals to avoid false positives occurring from multiple testing; accounting for uncertainty in model selection. Significant predictors for OS values were those for which 99% confidence intervals for the weighted average parameter estimates did not include zero.

The predicted values (Θ_i) from each model are dependent both on the magnitude of parameter estimates and on the relative weight ($_i W$) of each model including that parameter.

5.4 Results

5.4.1 Analysis of winter weather COE on adult OS and antioxidant defences in the following spring

5.4.1.1 Total antioxidant capacity - AOX

Winter body condition and PCrain were the only variables significantly associated with AOX at the 99% confidence level (Table 5.4). Because of the negative loading of rainfall on PCrain, this means that drier conditions were associated with higher antioxidant capacity. Higher body conditions were also associated with higher antioxidant capacity.

Table 5.4 Model averaging values for COE antioxidant capacity models, including relative importance of variables and 99% confidence intervals.

SQRT(AOX)	Importance	θ	Lower 99% CI	Upper 99% CI
Intercept		9.20*	6.81	11.59
Body condition	1.00	16.15*	8.14	24.16
PCrain	1.00	1.21*	0.75	1.67
PCtemp	0.10	-0.01	-0.13	0.11
Sex – Male	0.23	-0.01	-0.47	0.46

5.4.1.2 Oxidative damage - lipid peroxidation

Only winter PCtemp had a significant explanatory relationship with LP, with 99% CIs not overlapping zero (Table 5.5). Because of the negative loadings of temperature on PCtemp, milder winters were associated with higher levels of oxidative damage.

Table 5.5 Model averaging values for COE lipid peroxidation models, including relative importance of variables and 99% confidence intervals.

Log(LP)	Importance	θ	Lower 99% CI	Upper 99% CI
Intercept		2.45*	1.95	2.95
Body condition	0.68	-0.68	-2.38	1.03
PCrain	0.73	-0.07	-0.21	0.06
PCtemp	1.00	0.09*	0.03	0.15

5.4.1.3 **Peroxidase – antioxidant enzymes**

Only winter PCtemp had a significant effect on peroxidase levels, with 99% CIs not overlapping zero (Table 5.6). Because of the negative loadings of temperature on PCtemp, warmer winters were associated with higher spring peroxidase levels and thus better ability to cope with ROS.

Table 5.6 Model averaging values for COE peroxidase models, including relative importance of variables and 99% confidence intervals.

Log(PER)	Importance	θ	Lower 99% CI	Upper 99% CI
Intercept		-1.04	-1.21	-0.86
Body condition	0.31	0.10	-0.48	0.67
PCtemp	1.00	-0.08*	-0.11	-0.04

5.4.1.4 **Resistance to oxidative stress – RBC ½-life**

Only PCrain had a significant effect on RBC resistance to OS, with 99% CIs not overlapping zero (Table 5.7). Because of the negative loadings of rain on PCrain, this means that RBC ½-life in the following spring was longer with higher rainfall.

Table 5.7 Model averaging values for COE RBC ½-life models, including relative importance of variables and 99% confidence intervals.

Log(RBC)	Importance	θ	Lower 99% CI	Upper 99% CI
Intercept		4.16*	4.04	4.27
Body condition	0.69	0.19	-0.21	0.59
PCrain	1.00	0.03*	0.01	0.04
PCtemp	0.78	0.01	-0.01	0.03

In summary, wetter winters were associated with long RBC ½-life and lower antioxidant capacity. Warmer winters associated with higher peroxidase levels but also higher levels of oxidative damage.

5.4.2 Analysis of seasonal weather effects on cub and adult OS & antioxidant levels

5.4.2.1 Yearly cub survival probability

The three study years included different numbers of cubs trapped initially and different cohort survival rates. In 2012 a total of 41 cubs were caught, in line with the long-term population average (Macdonald *et al.* 2015a) with 20 (49%) surviving to adulthood (1 year old). In 2013, only 28 cubs were caught, with 12 (43%) surviving to adulthood. In 2014, although 60 cubs caught, only 22 (37%) survived to adulthood. Using these numbers, I infer that the small 2013 cohort reflects poor reproductive success and/or early pre-trapping mortality, indicative of a harsh year. I posit that those 12 cubs surviving to adulthood were likely to be 'good quality' individuals. In contrast, the 60 cubs caught initially in May 2014 was likely the product of mild, dry conditions in the spring; however, this cohort experienced substantial mortality later into the year.

A subset of 102 cubs was sampled for the analysis below, where the remaining cubs were too small to provide adequate blood samples in the spring.

5.4.2.2 **Antioxidant capacity – AOX**

PCtemp, season, Age-class*PCrain, Age-class*PCtemp, PCrain*season and PCtemp*season all had 99% CIs that did not overlap zero (Table 5.8). Higher temperatures were associated with higher AOX, particularly in the summer. Seasonally though, AOX was highest in the autumn, with the other two season approximately equal (Fig. 5.1). Increased rain in the summer had a positive association with AOX, but this association was negative in the autumn (Fig 5.2). Higher rainfall was associated with increased AOX in adults of both age classes compared to cubs (Fig 5.3). Old adults, however, had marginally lower AOX than cubs, when temperatures were high (Fig 5.4).

Table 5.8 Model averaging values for seasonal weather AOX models, including relative importance of variables and 99% confidence intervals.

SQRT(AOX)	Importance	Category level	θ	Lower 99% CI	Upper 99% CI
Intercept	0.93	-	15.43	12.59	18.28
Age-class	1.00	Cub	-	-	-
		Prime	-0.18	-1.64	1.28
		Old	-0.10	-1.57	1.38
BCI	0.90	-	4.33	-6.93	15.60
PCrain	1.00	-	0.56	-2.96	1.85
PCtemp	1.00	-	-3.43*	-5.02	-1.83
Season	1.00	Spring	-	-	-
		Summer	-74.36*	-117.48	-31.23
		Autumn	3.26*	1.93	4.59
Sex	0.19	Female	-	-	-
		Male	-0.03	-0.37	0.32
Age-class*PCrain	1.00	Cub*PCrain	-	-	-
		Prime*PCrain	1.11*	0.15	2.07
		Old*PCrain	1.48*	0.51	2.45
Age-class*PCtemp	1.00	Cub*PCtemp	-	-	-
		Prime*PCtemp	0.55	-0.10	1.21
		Old*PCtemp	0.88*	0.21	1.56
PCrain*Season	1.00	Spring/PCrain	-	-	-
		Summer*PCrain	6.41*	2.39	10.42
		Autumn*PCrain	-6.69*	-9.81	-3.57
PCtemp*Season	1.00	Spring/PCtemp	-	-	-
		Summer*PCtemp	-36.06*	-59.57	-12.56
		Autumn*PCtemp	NA	NA	NA

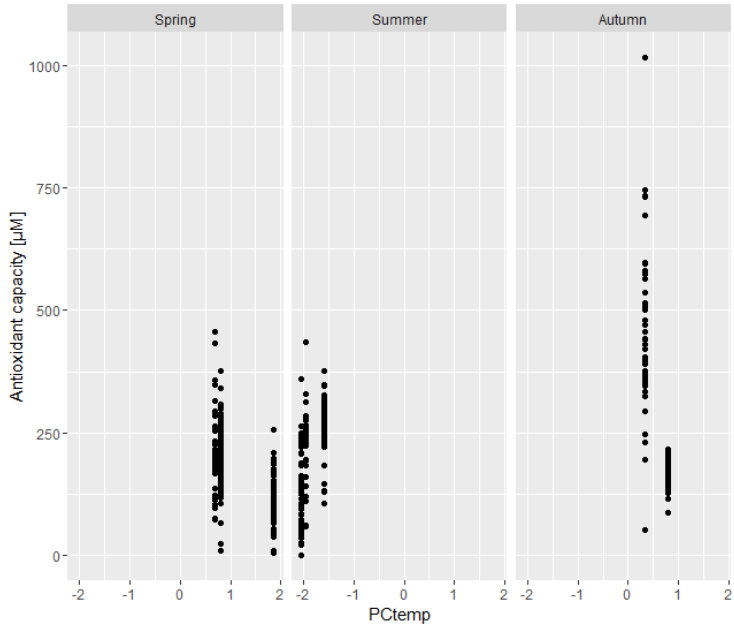


Figure 5.1The interaction of effects between PCtemp and season on antioxidant capacity.

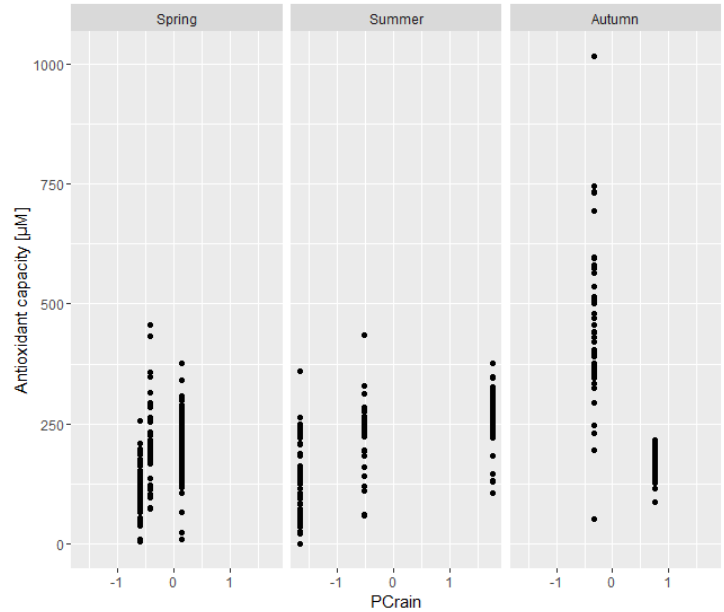


Figure 5.2 The interaction of effects between PCrain and season on antioxidant capacity.

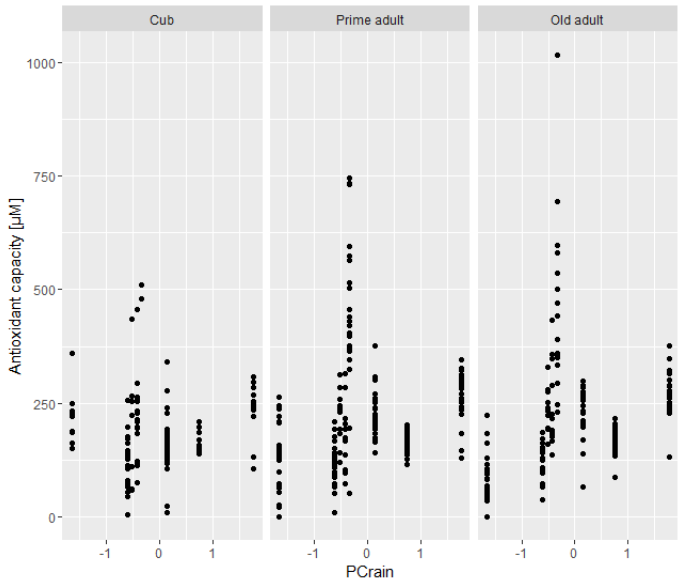


Figure 5.3 The interaction of effects between PCrain and Age-class on antioxidant capacity.

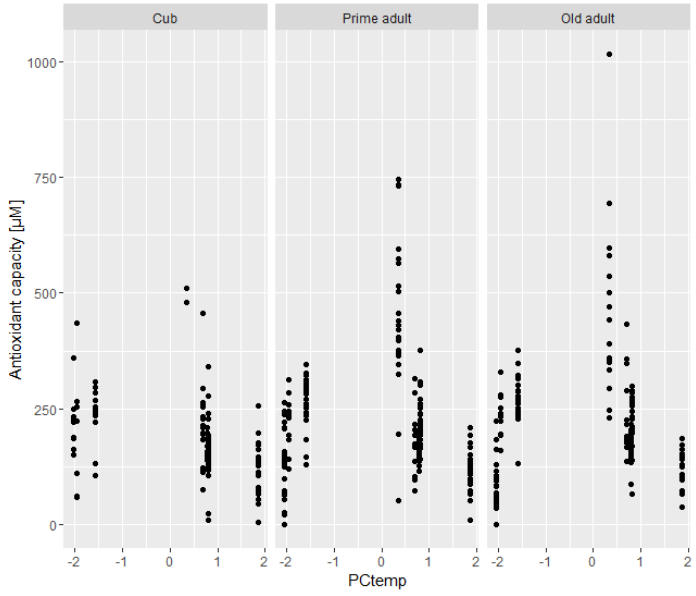


Figure 5.4 The interaction of effects between Age-class and PCtemp on antioxidant capacity.

5.4.2.3 **Antioxidant enzymes – peroxidase (PER)**

Age-class, PCrain, PCtemp, Season and the interactions Age-class*PCrain and PCrain*season all had confidence intervals that did not overlap zero (Table 5.9). Lower temperatures were associated with increased PER (Fig. 5.5). Rain had a positive association with PER in the spring but a negative association with PER in the summer and autumn (Fig. 5.6). Prime and old adults had higher PER levels than did cubs and this increased further in rainy conditions (Fig. 5.7).

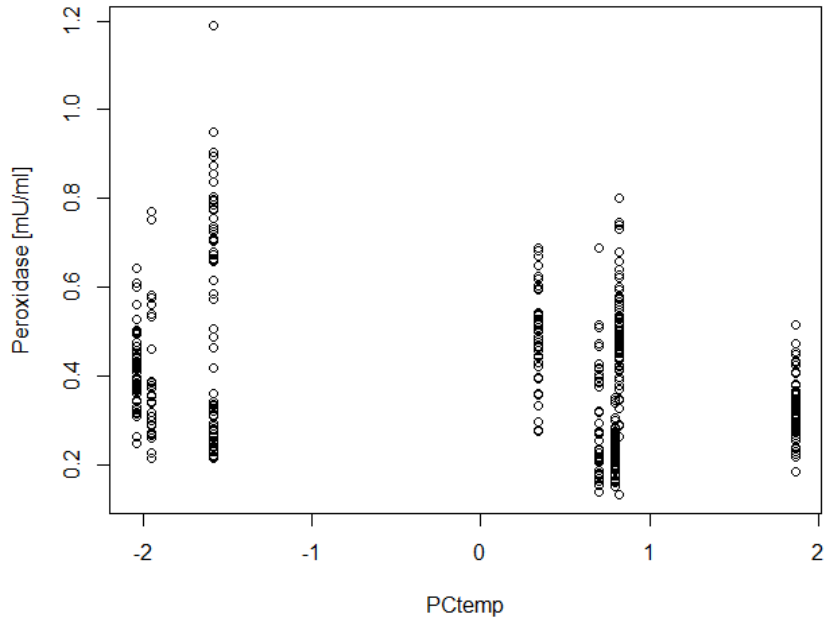


Figure 5.5 The effects of PCtemp on peroxidase levels.

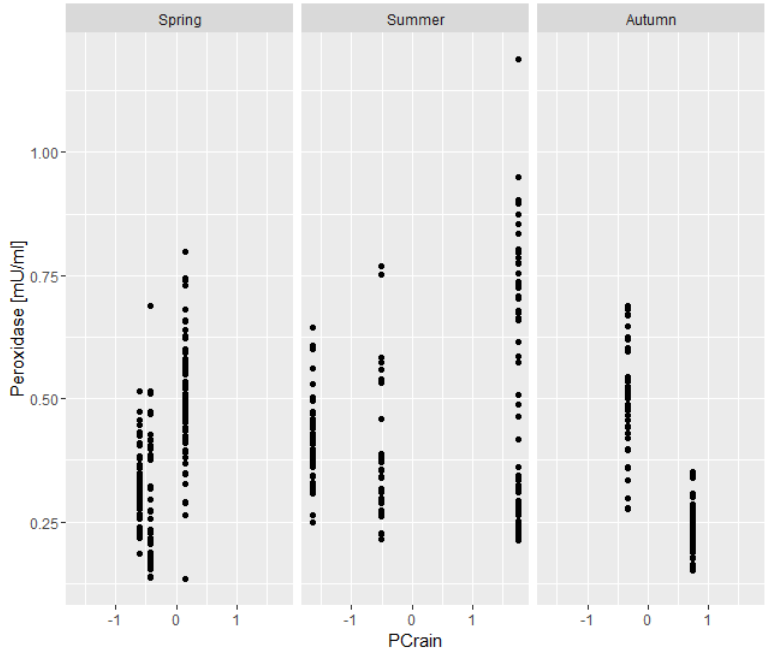


Figure 5.6 The interaction of effects between PCrain and season on peroxidase levels.

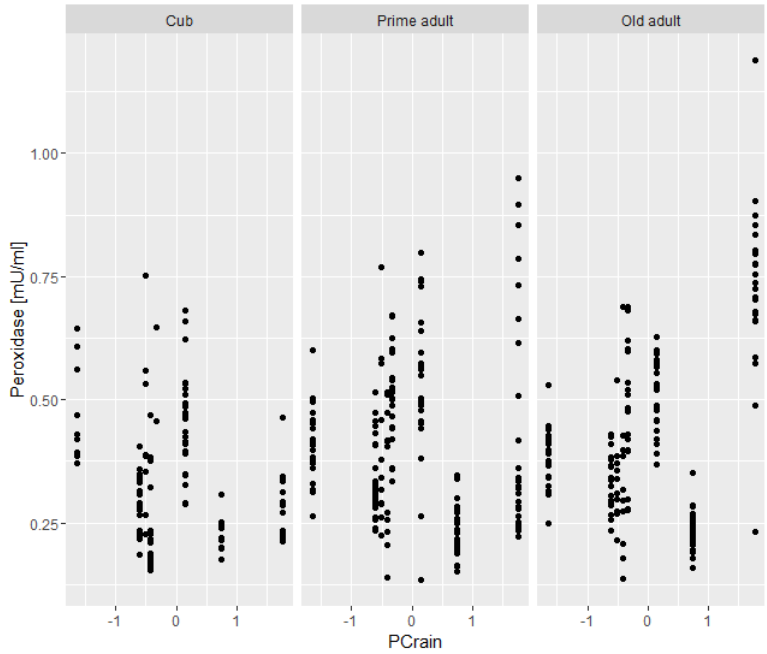


Figure 5.7 The interaction of effects between age-class and PCrain, on peroxidase levels.

Table 5.9 Model averaging values for seasonal weather peroxidase models, including relative importance of variables and 99% confidence intervals.

Log(PER)	Importance	Category level	θ	Lower 99% CI	Upper 99% CI
Intercept		-	-1.18	-1.38	-0.99
Age-class	1.00	Cub	-	-	-
		Prime	0.13*	0.02	0.24
		Old	0.18*	0.03	0.29
BCI	0.27	-	0.0007	-0.57	0.57
PCrain	1.00	-	0.84*	0.60	1.08
PCtemp	1.00	-	0.28*	0.13	0.43
Season	1.00	Spring	-	-	-
		Summer	4.88*	0.63	9.13
		Autumn	-0.03	-0.16	0.09
Age-class*PCrain	1.00	Cub*PCrain	-	-	-
		Prime*PCrain	0.10*	0.0001	0.20
		Old*PCrain	0.27*	0.18	0.37
PCrain*Season	1.00	Spring/PCrain	-	-	-
		Summer*PCrain	-1.29*	-1.68	-0.89
		Autumn*PCrain	-1.77*	-2.08	-1.47
PCtemp*Season	1.00	Spring/PCtemp	-	-	-
		Summer*PCtemp	2.28	-0.04	4.60
		Autumn*PCtemp	NA	NA	NA

5.4.2.4 **Oxidative damage – lipid peroxidation**

Age class, PCrain, PCtemp, Season, day temp and the interactions PCrain*season and PCtemp*season all had 99% CIs that did not overlap zero (Table 5.10).

Cubs had higher levels of LP than adults of any age (Fig 5.8). There was a small but significant effect of temperature the day before trapping on LP, where higher temperatures the day before trapping lead to (very) marginally higher values of LP (Fig. 5.9). Higher rainfall was associated with higher levels of LP in the summer and autumn compared to the spring (Fig. 5.10). Temperature was positively associated with LP with increased temperature in the summer further increased LP (Fig. 5.11).

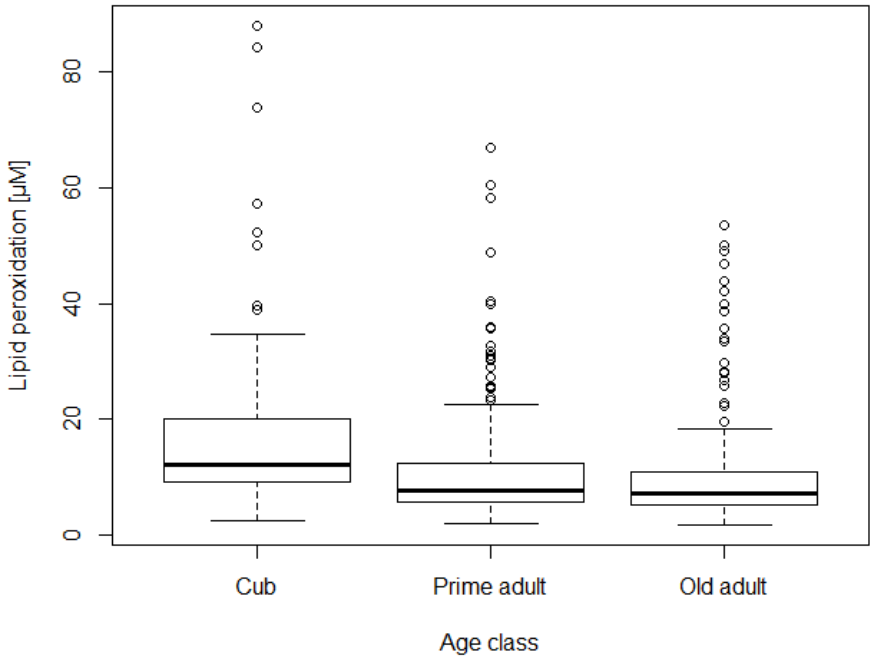


Figure 5.8 The effects of age-class on lipid peroxidation.

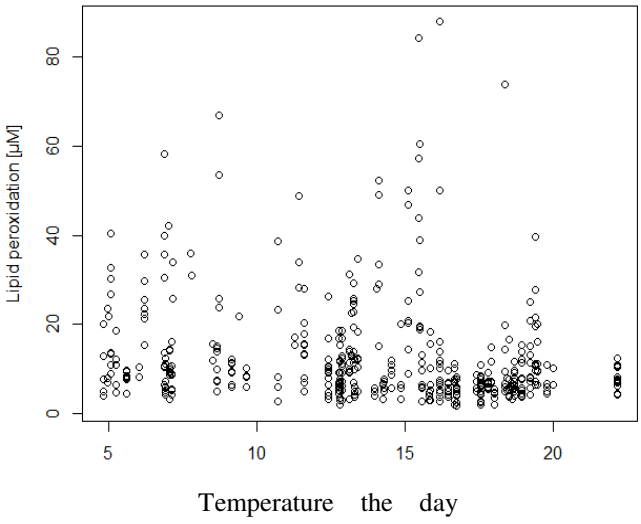


Figure 5.9 The effects of temperature the day before trapping on lipid peroxidation.

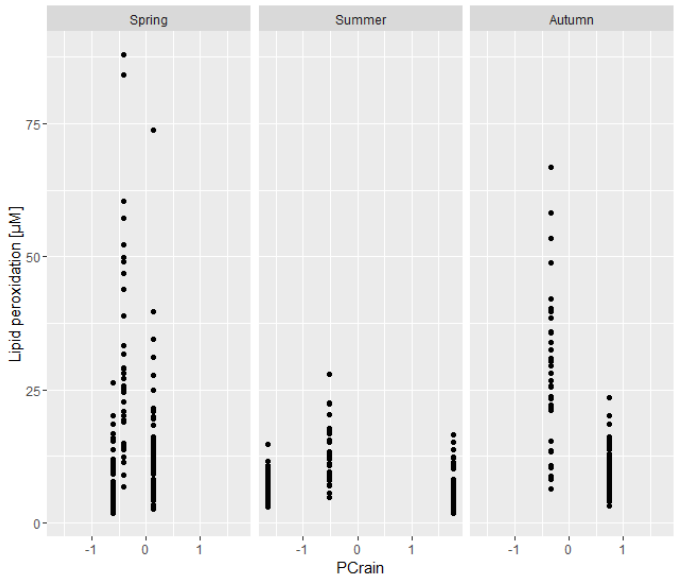


Figure 5.10 The interaction of effects of PCrain and season on lipid peroxidation.

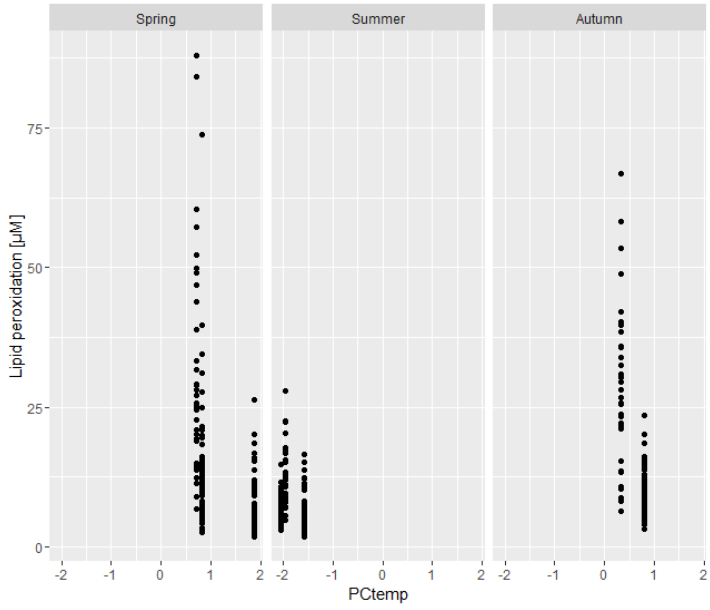


Figure 5.11 The interaction of effects of PCtemp and season on lipid peroxidation

Table 5.10 Model averaging values for seasonal weather lipid peroxidation models, including relative importance of variables and 99% confidence intervals.

Log(LP)	Importance	Category level	θ	Lower 99% CI	Upper 99% CI
Intercept		-	3.24	2.57	3.91
Age-class	1.00	Cub	-	-	-
		Prime	-0.36*	-0.56	-0.16
		Old	-0.42*	-0.63	-0.22
BCI	0.41	-	-0.15	-1.40	1.10
PCrain	1.00	-	-1.70*	-2.12	-1.28
PCtemp	1.00	-	-1.45*	-1.70	-1.21
Season	1.00	Spring	-	-	-
		Summer	-29.17*	-37.88	-20.46
		Autumn	0.46*	0.10	0.82
Dayrain	0.27	-	0.01	-0.02	0.03
Daytemp	1.00	-	0.05*	0.01	0.08
PCrain*Season	1.00	Spring/PCrain	-	-	-
		Summer*PCrain	3.65*	2.81	4.48
		Autumn*PCrain	1.26*	0.74	1.79
PCtemp*Season	1.00	Spring/PCtemp	-	-	-
		Summer*PCtemp	-13.52*	-18.25	-8.78
		Autumn*PCtemp	NA	NA	NA

5.4.2.5 **Resistance to oxidative stress – RBC ½-life**

PCtemp, season and the interactions PCrain*season and PCtemp*season all had 99% CI that did not overlap with zero (Table 5.11). Temperature was positively associated with RBC ½-life although older temperatures in the summer were associated with longer RBC ½-life (Fig 5.12). RBC ½-life was significantly shorter in the summer than in the spring. Increased rainfall was associated with longer RBC ½ -life in the summer and autumn compared to the spring (Fig.5.13).

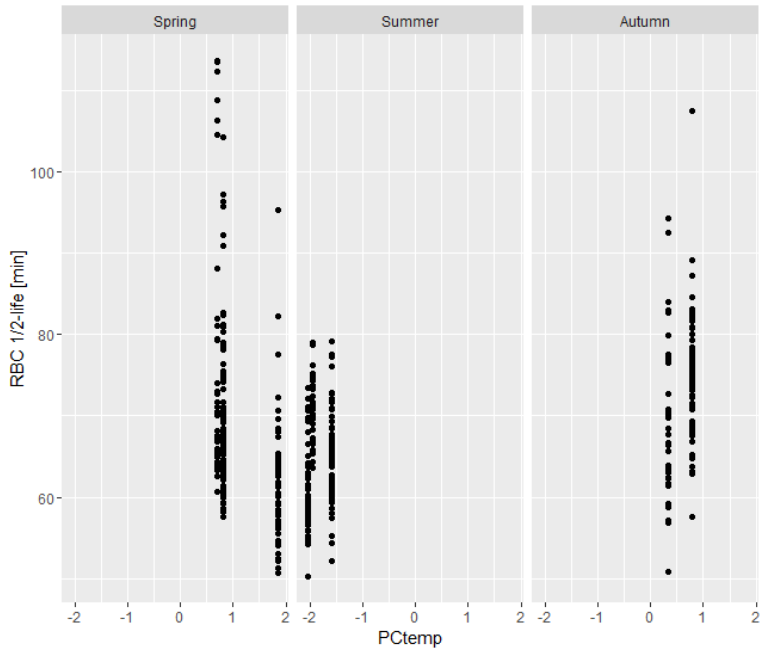


Figure 5.12 The interaction of effects of PCtemp and season on RBC ½-life.

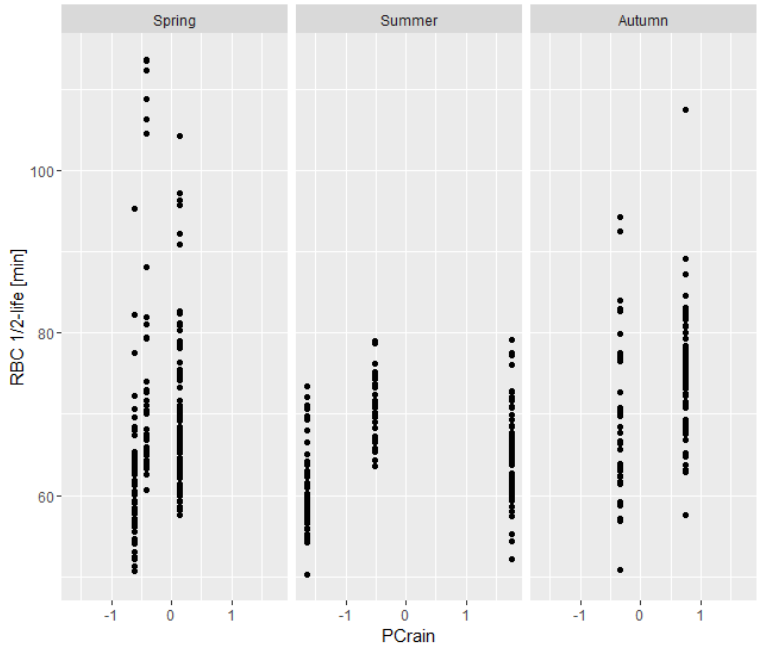


Figure 5.13 The interaction of effects between PCrain and season on RBC ½-life.

Table 5.11 Model averaging values for seasonal weather RBC ½-life models, including relative importance of variables and 99% confidence intervals.

Log(RBC)	Importance	Category level	θ	Lower 99% CI	Upper 99% CI
Intercept		-	4.36	4.12	6.40
BCI	0.29	-	0.06	-0.21	0.32
PCrain	1.00	-	-0.09	-0.21	0.03
PCtemp	1.00	-	-0.19*	-0.25	-0.13
Season	1.00	Spring	-	-	-
		Summer	-4.54*	-7.15	-1.95
		Autumn	-0.05	-0.18	0.08
Dayrain	0.65	-	-0.004	-0.01	0.004
Daytemp	0.29	-	0.003	-0.01	0.02
PCrain*Season	1.00	Spring/PCrain	-	-	-
		Summer*PCrain	0.422*	0.16	0.69
		Autumn*PCrain	0.25*	0.11	0.40
PCtemp*Season	1.00	Spring/PCtemp	-	-	-
		Summer*PCtemp	-2.15*	-3.57	-0.73
		Autumn*PCtemp	NA	NA	NA

5.5 Discussion

Ultimately, how populations cope with the stressors of climate change, is a function of how individuals respond to cumulative, contemporary weather stresses (Visser 2008, Noonan *et al.* 2014). It is thus clearly imperative to delve beneath broad-scale population and biogeographic responses, (e.g. Chen *et al.* 2011) to better understand the physiological mechanisms involved in coping with weather stress (Helmuth *et al.* 2005), and, particularly, the basis to differential, vulnerabilities of different age-classes (Newman and Macdonald 2015) – an approach thus far only taken in plant sciences (e.g., Pörtner 2002, Ahuja *et al.* 2010).

I found that carry-over effects (COE) from harsh winter conditions leave individual badgers in various levels of depleted 'physiological quality' going into the spring,

potentially affecting their ability to reproduce, care for cubs and compete for mating opportunities. Weather conditions in the three months preceding each measurement also affect physiological condition in both, cubs and adults, although given the different priorities between age-stages (simplistically: survival and growth vs survival and reproduction), weather conditions affect cubs and adults differently. Aside from a small effect of temperature the day before trapping on lipid peroxidation, there was no indication that short term weather (within 24hours of trapping) affected OS meaurements.

In wild animals, cachexia can occur when energy expenditure exceeds food intake (see Kock *et al.* 1992). This can arise due to seasonal paucity of food (summer drought / low winter productivity), demanding life-history events (e.g., juvenile development, dispersal, reproduction), or in tandem with disease (Chevin *et al.* 2010, Newman and Macdonald 2015).

Gomes-Marcondes & Tisdale (2002) report that oxidative stress contributes to muscle protein catabolism, causing tissue damage (as a build-up of malondialdehyde MDA). To mitigate OS during seasonal periods, when a lack of food can be anticipated, animals may pre-emptively consume foods rich in exogenous antioxidants and build up a reserve (see Catoni *et al.* 2008, Harrison *et al.* 2011).

5.5.1 Winter weather COE on adult OS and AOX capacity in the following spring

Following Harrison's (2011) suggestion that antioxidant capacity could be carried over from winter conditions, I demonstrate here a clear differential between the antioxidant levels of different adult badgers exhibited in spring, following food deprivation and

cachexia over the preceding winter. Warmer temperatures in the winter were associated with higher levels of lipid peroxidation, indicative of oxidative damage (Gutteridge 1995, Morrow *et al.* 1995). Drier winters were associated with high antioxidant capacity and longer RBC ½-life in the spring, while wetter winters were associated with high spring peroxidase levels. Badger cubs are born around the middle of February, and remain fully dependent on their mothers for the first 3 months (Macdonald *et al.* 2015a), depleting maternal BCI. In addition, badgers have delayed implantation (Hamlett 1935, Harrison 1963), where fatter females tend to implant more embryos around the shortest day of the year (ranging December 21st – 23rd; Woodroffe 1995). Thus, the condition of animals in the autumn will likely influence the probability of implantation and initial litter-size, while conditions over the winter will affect the outcome of the pregnancy. The condition in which females reach parturition, will further influence the quality of care and nutrition they will be able to provide to their cubs (see Chapter 4; Woodroffe and Macdonald 1995, Castillo *et al.* 2005).

In the UK, badgers do not truly hibernate (i.e., conserve protein during torpor, see Newman *et al.* 2011), but undergo varying extents of torpor, dropping their activity levels and basal metabolic rate with colder winter conditions (Noonan *et al.* 2014, McClune *et al.* 2015); a strategy to conserve energy and potentially reduce ROS production (Radak *et al.* 2008). Thus, low lipid peroxidation in colder winters suggests an effective tactical response, where torpor, reduced activity and metabolism lessen the risk of oxidative damage (Laidler 1984, Heldmaier and Ruf 1992). Not least, the Arrhenius equation dictates that the rates of – potentially deleterious – chemical reactions are slower at lower temperatures (Abele *et al.* 2002), although this also decreases enzyme activity rate which could compromise antioxidant defences (Kavanau

1950). This lack of activity in torpid badgers (Noonan *et al.* 2014) may reduce the risk of oxidative damage, but it also prevents foraging and the acquisition of nutrients (especially those rich in antioxidants; Alan *et al.* 2013), thus leading to low antioxidant capacity.

Antioxidant molecules and enzymes have slightly different functions (Mates 2000), but evidently warmwinter weather was primarily associated with high peroxidase levels, while drier weathers were associated with higher antioxidant capacity. Potentially, in warm conditions, where availability of resources such as AOX-rich earthworms are abundant, badgers are able to upregulate their antioxidant enzyme defences in an attempt to mitigate oxidative damage from thermogenesis (De Quiroga 1992, Wang *et al.* 2006) in order to maximise survival (Saino *et al.* 2011). Peroxidase enzymes often have a selenocystein in their active centre, requiring sulphur and selenium (Cao *et al.* 2012), which can be limiting in the environment (Rahmanto and Davies 2012). Easy access to worms in warmer, non-frosty conditions (Macdonald *et al.* 2015a) will maximise resource (and protein) acquisition (Zhenjun *et al.* 1997), and thus availability of these essential micronutrients, regulating peroxidase levels. Nevertheless, in other species (e.g. zebra finches;Eraud *et al.* 2007) it has been shown that cold acclimation can also result in adaptation of antioxidant enzyme regulation as well as mobilisation of antioxidant defences.

5.5.2 **Seasonal weather effects on cub and adult OS & antioxidant defences**

5.5.2.1 **Cubs**

Oxidative damage was highest in cubs. Growth rate and growth hormones are linked to high ROS production, resultant from metabolic demands (Holzenberger *et al.* 2003). A

high growth-rate often gives wild animals a maximum chance of survival in the initial phase of their life (Clutton-Brock *et al.* 1987, Beauplet *et al.* 2005), but this might come at the risk of increased OD, and thus risk of death later in development (Chapter 4). Indeed, zebra finches with higher growth-rates had lower RBC resistance to OS than did slower growing finches (Alonso-Alvarez *et al.* 2007b). Investment in initial growth rate, at the risk of compromising survival, should therefore be selected against in a K-selected species (Pianka 1970), where survival to the next year is typically important to maximise LRS.

In 2013, a small number of cubs were caught in the initial May trapping, and this cohort had unusually high AOX, equal to adult levels (see Bilham *et al.* 2013). In the summer of this same year, cubs had the highest peroxidase seen across all study years, where adults had the lowest. Potentially these results arose because adults were exhibiting signs of stress (Romero 2004), compared to the small number, yet good quality of surviving cubs (Folt *et al.* 1999). Highlighting the physiological quality of these cubs, the two surviving cubs sampled in the autumn of 2013 actually had lower levels of oxidative damage than most adults in the same season. In 2014 (and to a lesser extent 2012), I suggest that mild conditions allowed also for lower quality cubs to survive later in the year (Gaillard *et al.* 1997, Gaillard and Yoccoz 2003), thus yielding greater variability in all OS biomarkers. Simplistically, I propose that in mild conditions, individuals invest differentially in OS resistance and antioxidant defences, and are thus differentially vulnerable to conditions. In conditions of low resources, however, individuals all have to invest maximally in OS defences (see Alonso-Alvarez *et al.* 2006), potentially compromising reproduction (Alonso-Alvarez *et al.* 2004, Alonso-Alvarez *et al.* 2007a), investment in immunity (Bertrand *et al.* 2006), and social

communication such as subcaudal gland secretion (Buesching *et al.* 2003). There may, however, be a tipping point, where individuals are no longer able to cope with physiological stressors (Doak and Morris 2010, Nouvellet *et al.* 2013), especially in cases of energetically deficient animals (Fletcher *et al.* 2013). Nutritional conditions early in life can have long-lasting effects, where birds with low food quality early in life lead to lower antioxidant capacity later in life (Blount *et al.* 2003), these individuals are likely to be the ones to have a lower tipping point.

In 2013, summer rainfall was only half of the long-term mean (27mm vs 52mm). During dry summer conditions, badgers have short RBC ½-life, compared to intermediate rainfall years; this was potentially linked to starvation (see Sheridan and Collins 1983, Rios *et al.* 2005). Despite the oxidative danger associated with low antioxidant defences due to starvation induced cachexia (Godin and Wohaieb 1988), studies in artificially starved animals show that these markers return to normal following re-feeding (Boza *et al.* 1999, Morales *et al.* 2004). I thus expect badgers to return to less debilitated physiological condition as food availability increases.

Autumn conditions are critical for individuals to "fatten-up" in prevision of a harsh winter (Macdonald *et al.* 2010). Warmer, drier conditions in 2013 were associated with higher antioxidant capacity and oxidative damage than wetter, colder conditions 2012, which were also associated with high peroxidase levels. In relation to longer term climate data, the autumn of 2012 was notably wet (1.39 of 30 year mean) and colder than average (minimum temperature of 6.8°C compared to 7.5°C 30 year mean). Persistent precipitation causes badgers to remain below ground, in their sets (Noonan *et al.* 2014), and thus compromises their ability to forage and maintain energy budgets.

5.5.2.2 **Effects of body condition and sex on OS measurements in cubs and adults**

Only winter BCI was important for spring AOX, where higher BCI was linked to higher AOX. I found no evidence that individuals in good or bad body condition (BCI) showed differences in levels of OD or other antioxidant defences. This suggests that BCI, which is strongly linked to food availability only has a significant effect on aspects of physiology directly linked to nutrition such as non-enzymatic antioxidant capacity (Montes *et al.* 2011).

This is despite autumnal BCI being associated with activity regimes and weather conditions (Noonan *et al.* 2014). Additionally, BCI is associated with early cub survival (Chapter 4, Macdonald and Newman 2002), and OS levels varied with BCI when not taking into account weather conditions (Chapter 4). There is obviously a colinearity of predictor variables, where weather conditions affect BCI (Robson and Barriocanal 2008, Byrne *et al.* 2015) as well as OS.

Sex also had no distinct effect on OS metrics. Male badgers can have different testosterone strategies throughout the year, where some maintain high testosterone throughout the summer, while others do not (Buesching *et al.* 2009). Elevated testosterone is associated with higher risk of infection and more aggressive interactions with conspecifics (Braude *et al.* 1999), which would be expected to lower resistance to oxidative stress (Alonso-Alvarez *et al.* 2007a). Pregnancy and lactation in females, however, has a significant effect on oxidative damage levels (see Chapter 4; Castillo *et al.* 2005).

5.5.2.3 **Investigating cub development & phenotypes in relation to weather conditions**

Although distinct phenotypic responses to oxidative stress have been found for various traits in a diversity of species (reviewed in Metcalfe and Alonso-Alvarez 2010), cub OS measurements did not conform to any apparent discrete types. The annual cub cohort becomes increasingly diminished through selective mortality their first year (see Chapter 4, Laurenson 1994, Searcy and Sponaugle 2001, Collins and Kays 2011). Implicitly, therefore, cubs surviving later into the year (and / or to adulthood) form a sub-set of better quality (or luckier in terms of feeding success) individuals, which typically approached adult levels of antioxidant capacity, even in mild years (Fontagne *et al.* 2008). Especially in wet years, cubs had higher markers for oxidative damage, even though the temperatures were warmer. Because of their small size, cubs easily succumb to hypothermia (Nouvellet *et al.* 2013) , especially if their fur gets wet (Webb and King 1984, Campbell *et al.* 2013), and thus require a sufficiency of food to maintain elevated metabolic rates during harsh conditions (Chilliard *et al.* 1998). Although distinct phenotypes were not apparent, minimum inter-individual variation was observed in the harshest year (2013), potentially corroborating the initial hypothesis that all the "poor-quality" cubs died before the earliest opportunity to sample them. Conversely, in milder years, where a higher numbers of cubs survived until the spring trapping (2012, and especially 2014), there was great inter-individual variability in OS measurements, but along a continuum rather than in distinct phenotypes. This could suggest an interaction between the genetics driving survival and ROS production and the environmental conditions driving antioxidant capacity (Costantini *et al.* 2006, Olsson *et al.* 2008), supporting the hypothesis that while in good years a greater proportion of lower quality cubs survived until the first trapping, only a small

proportion of them (37% in 2014) survived to maturity. These factors will also interact with their coccidial infection status (Anwar *et al.* 2000). As clubs clear their infection during development, they will be able to absorb nutrients better, thus building up their antioxidant reserves (Newman *et al.* 2001).

5.6 **Conclusion**

Assessing responses to climatic change trends requires long-term data, and generally lies outside the scope of typical research study periods, including this one. Detecting ecological or physiological responses to prevailing, or cumulative, weather effects is, however, more achievable. Nevertheless, Noonan *et al.* (2015b) caution that because of inter-annual variation in weather and correlative variation in behavioural and / or physiological responses, short-term studies are more prone to skew than longer-term research, potentially biasing interpretation. Capturing the temporal scale of variability under investigation is also essential. Even a 20 year study recording how individuals respond to a succession of completely average years will be less informative than required; clearly years including extreme weather are necessary to look at the ultimate extent to which conditions drive adaptation and / or cause natural selection. Despite these constraints I still found that inter-annual deviations in weather from normative values exacerbated both seasonal and age-class specific oxidative stress in manifest ways. Thus, when looking at the gross effects of climate change on a species' biogeographical distribution and population dynamics (e.g. Walther *et al.* 2002, Parmesan and Yohe 2003), it is imperative to understand the mechanisms underlying these phenomenon.

Crucially, the need for this understanding is also not simply a scholarly endeavour. As anthropogenic factors alter conditions for wild populations (White and Ward 2011, Sih

2013), a much more detailed understanding of the mechanisms involved is essential for understanding how individuals will be able to cope with HIREC, and whether current adaptive mechanisms and plasticity will be sufficient for species to survive.

Chapter 6: **Development of molecular and immunological methods for the investigation of innate immunity in the European badger in the absence of genomic resources**

6.1 **Introduction**

While ecologists have, historically, explored scientific questions through phenomenology, attributing causes to observed effects, adopting a molecular approach offers greater specificity to the research (Hughes 1998, Morin *et al.* 2004, Baker 2009). At the interface of these complementary disciplines is the need to establish efficient genetic markers for population biology (Sunnucks 2000, Thomson *et al.* 2010) or targets for molecular analyses of gene expression.

This has led to the emergence of new fields such as eco-physiology, immuno-ecology and conservation genetics. These fields are united by solving field-ecology questions using laboratory techniques, such as in population pedigree (Dugdale *et al.* 2008, Annavi *et al.* 2011), phylogeny (e.g., Murphy *et al.* 2001), heredity (e.g.Doff *et al.* 1991, Goudet *et al.* 2002), gene flow (e.g., Rutledge *et al.* 2010) and introgression (e.g., Bohling *et al.* 2013). In this context, quantitative PCRs are used to investigate gene expression to determine how differently adapted individuals respond to changes in environmental conditions (Coltman *et al.* 1996, Bockelmann *et al.* 2013). These approaches, however, require defined gene sequences, which are often lacking when genomes are not available. Thus, techniques need to be developed to expand the existing work, and develop molecular methods for organisms for which genomes are currently not available.

Due, in substantial part, to a lack of genomic resources for wild-living species (versus laboratory model species), advances in immuno-ecology have been limited. In contrast to laboratory based immunology, immuno-ecology aims to explain how natural variation, arising through biotic and abiotic factors, contributes to differences in immune functions (Sheldon and Verhulst 1996, Schmid-Hempel 2003, Schulenburg *et al.* 2009, Bordon 2016). Furthermore, immune responses are influenced by the sex, age, health and nutritional status, of focal species, in the context of natural selection and evolutionary ecology (Martin *et al.* 2011). In turn, this requires the development of specific techniques to explore the effects of variation. To bridge this deficit, to date many immuno-ecological studies have focused on wild rodent systems, for which genomes of closely related species are available, or resources allow experiments using murine reagents that cross-react (Abolins *et al.* 2011, Babayan *et al.* 2011, Pedersen and Babayan 2011, Viney *et al.* 2015).

The immune system is complex and involves a wide range of cell types and molecules to mediate anti-pathogen responses. Hence, to define wildlife immune responses requires the identification of relevant genes to describe immune responses (Boughton *et al.* 2011). This is of particular importance for the investigation of wildlife species that are reservoirs for epizootic and zoonotic diseases, requiring management or conservation intervention (Deem *et al.* 2001), for which specific immune responses and gene upregulation need to be investigated. In the absence of species-specific genomes, many studies use homologous or orthologous genes (i.e. the same gene from a closely related species: Gabaldón and Koonin 2013, which can be found using GenBank, the main gene repository; Benson *et al.* 2013) in order to develop molecular assays (Ranwez *et al.* 2007, Sin *et al.* 2012b, Pechouskova *et al.* 2015). Using available

genomes and assays for species that are closely related in order to carry out assays on the species of interest (Pechouskova *et al.* 2015), however, can cause potential issues with the specificity of the assays as they are originally developed for different species. In the case of molecular research investigating the particular workings of a study animals' immune system, or the investigation of functionality of genes and conservation of secondary gene structure, it is important to develop assays that are specific to the study animal. In addition, it is crucial to be working with highly specific primers when quantifying upregulation of genes, so that specificity of primers is not a biasing factor in calculating gene copy numbers (see Peters *et al.* 2004). The information available on orthologous genes, such as annotations can then be used to inform analysis of newly sequenced genes of the target species. The process of sequencing new, species-specific genes requires them to be assembled and then checked in the context of phylogenies (Odbileg *et al.* 2005), structure and function in order to characterise the similarities and differences from orthologous genes. While finding differences between species is inevitable, in many cases conservation of structure and function is a result in itself (Viney *et al.* 2015), on which to build further studies.

New methodology can be required in many studies, but rapid development of species-specific assays is crucial for dealing with the emergence of zoonotic and epizootic diseases. These diseases can cause significant financial and ecological / conservation damage (Gillespie *et al.* 2008, Karesh *et al.* 2012, Narrod *et al.* 2012), often requiring thorough investigation of the immune system of the species of interest to find a solution to the problem. In this chapter, I develop a methodology which could be applied to a wide range of wildlife species to identify immune genes, verify their functionality and

investigate the initial recognition of pathogens and response via the innate immune system.

In this context, the European badger provides a tractable wildlife model, because in the UK it occurs at high densities (Johnson *et al.* 2002a) and is implicated in the maintenance and transmission of bovine tuberculosis (bTB) to cattle (Woodroffe *et al.* 2006, Reilly and Courtenay 2007). Many studies have considered ecological factors linked to infection status (Vicente *et al.* 2007, Corner *et al.* 2012) and transmission risks (Carter *et al.* 2007), with consequences for vaccination strategies (Lesellier *et al.* 2009a, Carter *et al.* 2012b). Immunological studies of the badger-bTB disease system, using molecular methods, have been fewer (Buddle *et al.* 2000, Gormley and Costello 2003), but include the involvement of MHC complex (Sin *et al.* 2012a, Sin *et al.* 2012b), leukocyte coping capacity (Montes *et al.* 2004, Montes *et al.* 2011), and Interferon gamma (IFNγ, Dalley *et al.* 2004a, De la Rua-Domenech *et al.* 2006, Beirne *et al.* 2016). Nevertheless, fundamental research into the functioning of the badger's immune system, and how it responds to pathogens, is lacking. Importantly, although critical to bTB management, no work has been done to investigate (innate) immunological response to *Mycobacterium* infection (Chapter 7), despite this being a key line of defence (Kleinnijenhuis *et al.* 2011).

A major constraint on work in the badger is that there is currently no badger genome, and very few individual gene sequences are available (but see Carpenter *et al.* 2003, Dalley *et al.* 2004a, Annavi *et al.* 2011), especially in the area of innate immunity.

Here, I develop methods allowing us to investigate how badger macrophages recognise pathogens, with particular focus on TB, through Toll-like receptors, one of the main

groups of pathogen recognition receptors (PRRs), and how this elicits an initial immune response. The final objectives (Chapter 7) were to quantify the production of various pro-inflammatory cytokines in blood derived monocyte-macrophages, in response to stimulation with Toll-like receptor agonists, including multi-TLR agonists such as TB. The current chapter will deal specifically with the development and validation of an array of molecular assays, as well as the information which can be drawn from such methods. It will also include a brief analysis of gene structure and sequences to investigate any potential differences in gene function. The results of the experiments described in this chapter will be treated in more detail in Chapter 7.

Specfically, I aim to:

1) Identify badger genes, sequence them and put them in the phylogenetic context of other genes and species (using phylogenetic trees, sequence alignments and investigating conservation of structural domains) to ascertain their functionality and percentage identity to other species.
2) Develop qRT-PCR primers and assays for badgers in order to investigate the regulation of various immune genes following TLR stimulation. Use time courses to identify the best time points to use achieve this, taking into account field constrains.
3) Stimulate badger macrophages with various TLR agonists and measure cytokine levels by qRT-PCR in order to identify how badgers respond to various TLR agonists (including TB) and compare with other species.

6.2 Methods

6.2.1 Extraction of badger cDNA from white blood cells

6.2.1.1 Cell isolation and culture

Blood samples were collected via jugular venepuncture into lithium heparin vacutainers (*BD Biosciences*) and centrifuged immediately (1500 g, 10 min, 4°C) to isolate plasma. White and red blood cells were resuspended in culture medium until extraction of white blood cells could take place. Peripheral blood mononuclear cells (PBMCs) were isolated following established procedures (Wigley *et al.* 2002). Briefly, cells were isolated using Ficoll (Life Technologies), washed and re-suspended in culture medium comprising phenol red-free Dulbecco's Modified Eagle Medium (Life Technologies) containing 10% FCS (Foetal Calf Serum, Life Technologies), 2 mM Penicillin and Streptomycin (Life Technologies), and 2 mM L-Glutamine (Sigma-Aldrich; chapter 2).

6.2.1.2 RNA extraction and cDNA synthesis

RNA was extracted from cells frozen in RLT buffer (Qiagen) using either single columns or 96 well format columns (both Qiagen) following the manufacturer's spin protocol. For qRT-PCR, the input RNA amount was normalised for cDNA reactions across a sample set and cDNA was generated (Life Technologies).

6.2.2 Identification of badger immune genes

6.2.2.1 Badger gene sequences

Primers were designed based upon regions of target genes conserved in multiple mammalian genomes (Table 6.1 for primers, Supplementary 1 for alignments). Appropriate primer sets were designed and used to amplify fragments of badger cytokine or TLR genes from PBMC cDNA using Myfi PCR mix (Bioline), according to

manufacturer's instructions. Products were cloned into pGEM TEasy (Promega) and sequenced using plasmid targeting (M13; Royo *et al.* 2007) or gene specific primers with BigDye chemistry (Life Technologies), according to the manufacturer's instructions. Sequences were analysed using Bioedit (Hall 1999), and badger-specific gene sequences used to design primers for 5' RACE PCR. 5' RACE. cDNA was generated using a SMARTer RACE kit (Clontech) according to the manufacturer's instructions. PCR products generated from RACE PCR products were cloned into pGEM TEasy, as described, or into pTarget (Promega) if longer than 1 kb, and sequenced as above. All sequences were aligned using Clustal Omega (Sievers *et al.* 2011) and refined manually, with trimming to remove unaligned or poorly aligned stretches at the N and C termini. The sub-alignment of iNOS sequences was formatted using BioEdit 7.2 (Hall 1999) and CorelDRAW X5.

Domains were identified in our gene sequences using SMART (Letunic *et al.* 2015). TLR leucine rich repeats, transmembrane domains and TIR domains were also identified manually based on Matsushima *et al.* (2007), and similarity with Genbank annotations on related species sequences investigated (Benson *et al.* 2013). Alignments in FASTA format are provided within Supplementary data 1 (NOS & TIR domain). Alignments for IL1β, IL6, IL12p40 and TNFα are provided in Supplementaty data 1.

Table 6.1. RT-PCR primers, based on Svitek and von Messling (2007) or designed manually

Gene	Primers	
	Forward	Reverse
IL1β	5'GAATCTGTACCTGTCCTGTGTGAT-3'	5'-CTGAGAGGTGCTGATGTACCAG-3'
IL6	5'-CAAATGTGAAGACAGCAAGGAGGCA-3'	5'-TCTGAAACTCCTGAAGACCGGTAGTG-3'
IL12p40	5'-ATCGAGGTTGTGGTGGGTGCTATT-3'	5'-TAGGTTCATGGGTGGGTCTGGTTT-3'
TNFα	5'TGGAGCTGACAGACAACCAGCTAA-3'	5'-TGATGGTGTGGGTAAGGAGCACAT-3'
IFNγ	5'- CCATCAAGGAAGACATGCTTGTCAGG-3'	5'- CTGGACCTGCAGATCATTCACAGGAA-3'
iNOS	5'- CTTGCAAGTCCAAGTCTTGTCTG-3'	5'-CCTGGCCAGATGTTCCTC-3'
TLR2	5'- CTTTTATTTCCCTGCGGAGTC-3'	5'- TTCCCCGAGGGATTTGTAAC-3'
TLR9	5'-CAGCTGCTGCCTAGTTTGC-3'	5'-TGCACCAGGAGAGACAAGG-3'
GAPDH	5'-AACATCATCCCTGCTTCCACTGGT-3'	5'-TGTTGAAGTCGCAGGAGACAACCT-3'

6.2.3 **Development of qRT-PCR assays**

Sequences were compared to human, mouse, dog and ferret sequences to identify target regions for intron spanning qRT-PCR assays using SYBR green technology (Table 6.2). Assays were tested for specificity by melt curve analysis, and cloning and sequencing of amplicons. Validated assays were then used to analyse cytokine cDNA levels in agonist treated PBMC. Dilution series of linearised plasmids containing cytokine or TLR gene sequences were used to calculate copy numbers. Ct values for TLR or cytokine target amplification for each sample were adjusted using the GAPDH Ct value for the same sample, to account for variation in sampling and RNA preparation. The slopes of a plot of Ct against $\log_{10}$ of the plasmid dilution series present on each plate were calculated. The slopes of the respective gene of interest (GOI) and GAPDH dilution series were used to calculate GOI Ct values and adjusted for differences in

input total RNA as follows: corrected Ct value = Ct + (Nt – Ct') 3 S/S', where Ct is the GOI Ct value, Nt is the median GAPDH Ct for all samples within an experiment, Ct' is the GAPDH value of the individual sample, S is the GOI slope, and S' is the GAPDH slope (Wu *et al.* 2010).

Table 6.2 Badger qRT-PCR primers, designed from cloned badger sequences, and in most cases (except for iNOS), intron spanning.

Gene	Primers	
	Forward	Reverse
IL1β	5'-CTGTACCTGTCCTGTGTGATGAAAG-3'	5'-GTTATGTCCTGACCACCTCTGTTATTTCC-3'
IL6	5'-GAACTCCCTCTCCACAAGCG-3'	5'-GCCAGTGCCTCCTTGCTG-3'
IL12p40	5'-CATTTGGGAACTGGAGAAAA-3'	5'-ACCGGAGCCTAAGACTTCAC-3'
TNFα	5'-CCTGCTGCACTTTGGAGTGATC-3'	5'-CTATTAGCTGGTTGTCTGTTAGCTCCAC-3'
IFNγ	5'-GAAGAACTGGAGAGAGGAGAGTGAC-3'	5'-CCGCTTACTGCTGCTGCTATTG-3'
iNOS	5'-CCTGTGTTCCACCAGGAGAT-3'	5'-CTTCTCCGGGGTCTCTTCTT-3'
TLR2	5'-CTTTTATTTCCCTGCGGAGTC-3'	5'-TTCCCCGAGGGATTTGTAAC-3'
TLR9	5'-CAGCTGCTGCCTAGTTTGC-3'	5'-TGCACCAGGAGAGACAAGG-3'
GAPDH	5'-AACATCATCCCTGCTTCCACTGGT-3'	5-AGGTTGTCTCCTGTGACTTCAACA-3'

A 10-fold dilution series of linearised plasmid DNA was prepared for each target, starting at 20 million copies. Ct values for the dilution series were calculated as described for samples. Plots of 40 – Ct against copy number of the serial dilution were used to derive sample copy numbers per well using the following equation: sample copy number = $y*e^{(ab)}$, where "y" is the y intercept, "a" represents the slope of the dilution series and "b" is the corrected 40-Ct of the sample.

6.2.4 Investigating the upregulation of immune genes in response to TLR stimuli

Cells were plated at 5 x 10^5 cells/well on 96 well plates, and either treated or mock-treated with defined TLR agonists or microbial lysates (all obtained from Invivogen, concentrations given in Table 6.3). For the initial time course, cells were stimulated at 2, 4 and 8 hours. For TLR agonist induced cytokine analyses, cells were stimulated for 4 hours (8 hours to cross check timings). For the final TB time course, cells were stimulated at 2, 4, 8, 12 and 24 hours.

Table 6.3 Agonist treatment of badger macrophages. The table shows the targeted TLR, the compound used, with its abbreviation and the concentrations it was used at.

TLR targeted	TLR agonist	Concentration
TLR 2	Pam3CSK4 (PAM)	300 ng/ml
TLR 2	Lipoarabinomannan from *M. smegmatis* (LAM)	1 µg/ml
TLR 3	Poly (I:C) (PIC)	12.5 µg/ml
TLR 4	Lipopolysaccharide (LPS)	1 µg/ml
TLR 5	Flagellin (FGN)	10 µg/ml
TLR 7	R848/Imiquimod	1 µg/ml
TLR 9	ODN m362	5-100 µg/ml
TLR 9	*E. coli* DNA	5-100 µg/ml
Multiple	Heat killed *Mycobacterium tuberculosis* (HKTB)	1 µg/ml
Multiple	Heat killed *Listeria monocytogenes* (HKLM)	10^7-10^8 cells/ml
Multiple	Heat killed *Mycobacterium bovis*	10^7-2x10^8 cells/ml

6.2.5 Making recombinant IFNγ for macrophage stimulation

Sequence encoding full-length badger IFNγ (including signal peptide), and an 8 residue C-terminal sequence encoding a flexible linker (GG) and a polyhistidine affinity tag (HHHHHH) were ordered as a synthetic gene. The synthetic gene was supplied with flanking BamHI and NotI restriction enzyme recognition sites, and so BamHI-HF and

NotI-HF (New England Bioscience) were used to sub-clone it into the pTARGET mammalian expression vector. This construct was prepared in endotoxin-free form using the Endofree Maxi kit (Qiagen), and transfected into serum-free media-adapted HEK292T cells (serum-free medium used was Freestyle, from Life Technologies). Transfections were performed using TransIT-2020 (Mirus), and cells were cultured for three days before the supernatant was harvested, spun to remove all cellular debris, and concentrated ~100-fold using a 20K MWCO spin concentrator (Pierce). Polyhistidine-tagged protein was then assayed by dot blot: a serial dilution of the concentrated sample (and of a control supernatant following transfection with an empty plasmid) spotted on to nitrocellulose membrane, alongside a dilution series of a pure polyhisitidine-tagged protein of known concentration. The dried membrane was blocked (StartingBlock PBS with Tween, Pierce), and probed with 100ng/ml anti-penta-his (Qiagen), followed by anti-mouse IgG-HRP (Sigma). Between each incubation, extensive washing with PBS / 0.1% Tween was carried out. Dots were visualised using ECL reagent, and concentrations estimated by reference to the protein of known concentration using densitometry in ImageJ (Fig 6.1).

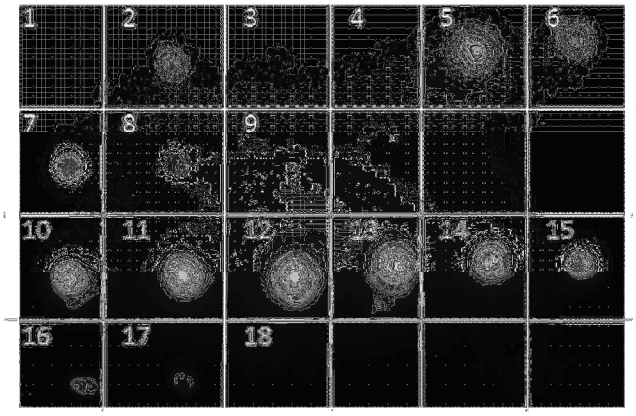


Figure 6.1. Quantification of recombinant IFN-γ

1. 293T/EGFP supernatant (sn) (pre-concentration) 2. 293T/badger IFNγ sn (pre-conc.) 3. 293T/EGFP sn (conc.) 4. 293T/EGFP sn (conc.), diluted 1/4 5. 293T/ badger IFNγ sn (conc.) 6. 293T/ badger IFNγ sn (conc.), diluted 1/4 7. 293T/ badger IFNg sn (conc.), diluted 1/16 8. 293T/ badger IFNγ sn (conc.), diluted 1/64 9. 293T/ badger IFNγ sn (conc), diluted 1/256 10. Japanin-his (Jap-h; positive control protein, for standard curve), 1µg 11. Jap-h, 500ng 12. Jap-h, 250ng 13. Jap-h, 125ng 14. Jap-h, 62.5ng 15. Jap-h, 31.2ng 16. Jap-h, 15.6ng 17. Jap-h, 7.82ng

6.3 **Results and Discussion**

Because the gene-sequencing process is, by definition, a step-wise one, defined by previous results that were obtained along the way, the results and discussion will be combined in order to explain each result in turn, and how it informed the next logical steps.

The easiest way of assessing macrophage reactions to TLR stimulation is by measuring the production of nitric oxide (assayed as nitrite) in response to the stimulation of cells with immuno-stimulatory molecules (TLR agonists or IFNγ) using Griess reactions (Fox Jr 1979, Sun *et al.* 2003). Thus, initial immunological work on badgers was performed using these simple methods. The results of these experiments will be presented in more detail in Chapter 7, but briefly, stimulation of badger macrophages did not result in production of nitric oxide through TLR stimulation or treatment with recombinant IFNγ. In the face of this unexpected result, I thus needed to develop

molecular assays to assess the upregulation of cytokines and iNOS in badgers (e.g. Q-RTPCR).

6.3.1 **Identification of badger genes**

The first step was to identify homologues / orthologues of the relevant genes in different species, align them and identify regions of homology. Two of the closest species to badgers that have full genomes available are the dog and ferret. Once the respective regions were identified, primers were designed based on the ferret sequence and the conserved regions of homology between these species, as well as homology with mouse and human (Table 6.1; Svitek and von Messling 2007).

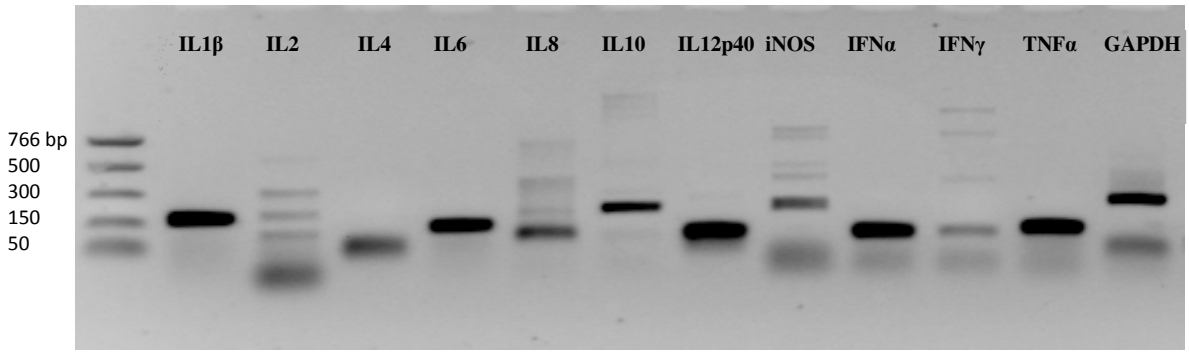


Figure 6.2. RT-PCR using pooled badger cDNA products run on a 1% agarose gel

In Figure 6.2, correct sized bands were amplified for IL1β, IL4, IL6, IL10, IL12p40, IFNα, TNFα and GAPDH. Expected fragment sizes were obtained for IL2, IL8, iNOS, and IFNγ, but with additional bands at other sizes, suggesting primers are not only amplifying the desired product, but also some other fragments.

In order to focus on the pro-inflammatory cytokines and TLRs of interest for their links to TB recognition, I limited further analysis to the following genes: TNFα, IL1β, IL6, IL12, iNOS, GAPDH, INFγ, IFNα (which was dropped further in the analysis, see below), TLR2 and TLR9.

PCR reactions for the correct length fragments were cleaned up, cloned and sequenced; which verified the identity of the cloned gene fragment using BLAST (Boratyn *et al.* 2013). For those genes where more than one band was present, I cut out the piece of the gel corresponding to the expected length and cloned and sequenced that fragment. Once badger-specific sequences had been obtained, I were able to redesign primers so that they were specific to the European badger. These were again verified by PCR and cloning. Most genes were cloned in small fragments and assembled into the full length sequences. iNOS, however, proved difficult to clone, and it took a combination of cDNA, genomic DNA and RACE cDNA to assemble the full length sequence, although badgers were confirmed to carry a form of this gene in their genome (see Chapter 7).

Quantitative RT-PCR primers were also designed based on the badger sequences obtained (Table 6.2). In order to determine copy numbers, rather than fold change, I developed plasmid standards with known gene copy numbers. In this way, I were able to investigate whether the increase in expression was likely to have any kind of biological significance. The qRT-PCR program and primer concentrations were optimised in order to obtain correctly shaped melt curves (Fig. 6.3). Titrations of each primer were performed in order to determine the ideal concentration for each gene based on the ability of the primers to detect the target without creating significant primer dimers.

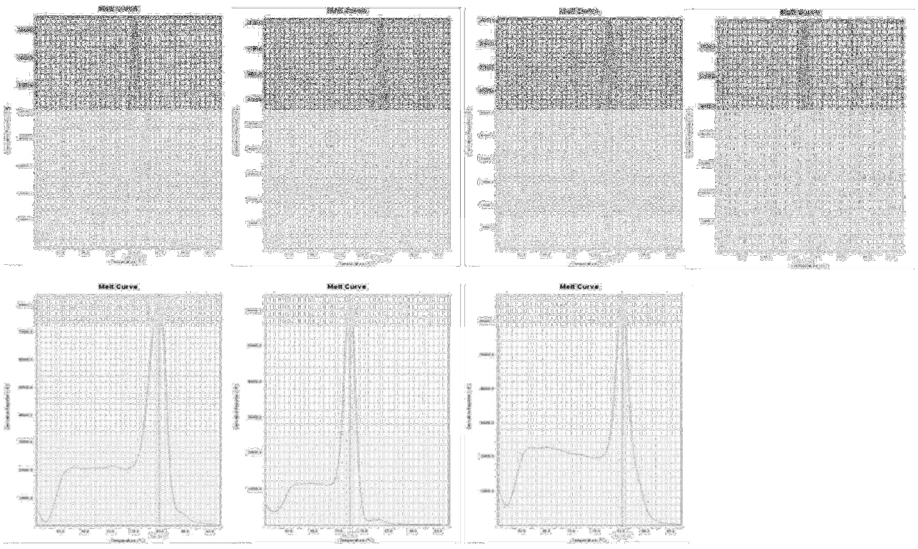


Figure 6.3: qRT-PCR melt curves

(L-R, top): IL1β, IL6, IL12p40, IFNγ, (L-R, bottom) iNOS, TLR2, TLR9 and TNFα.

6.3.2 **Alignments and domain conservation of badger immune genes**

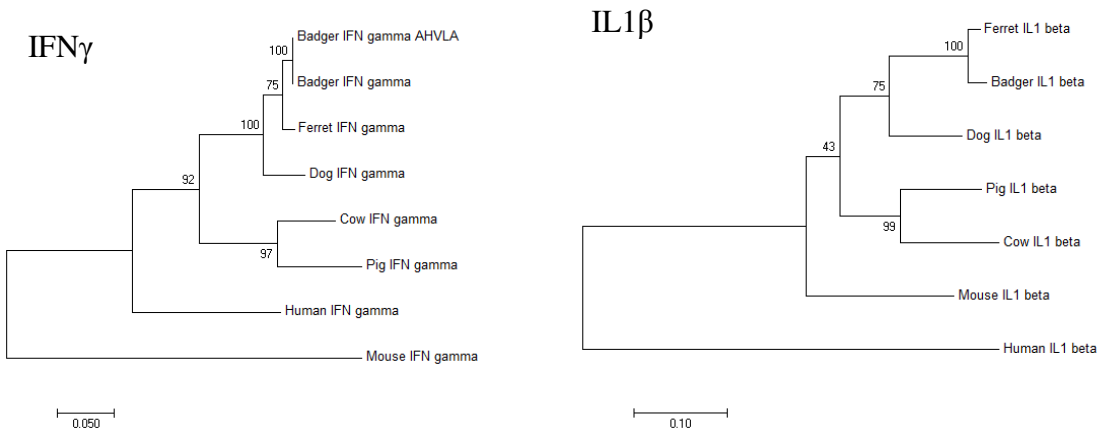
All the target badger gene sequences detected were orthologues of the genes in other species (Supplementary data 1).

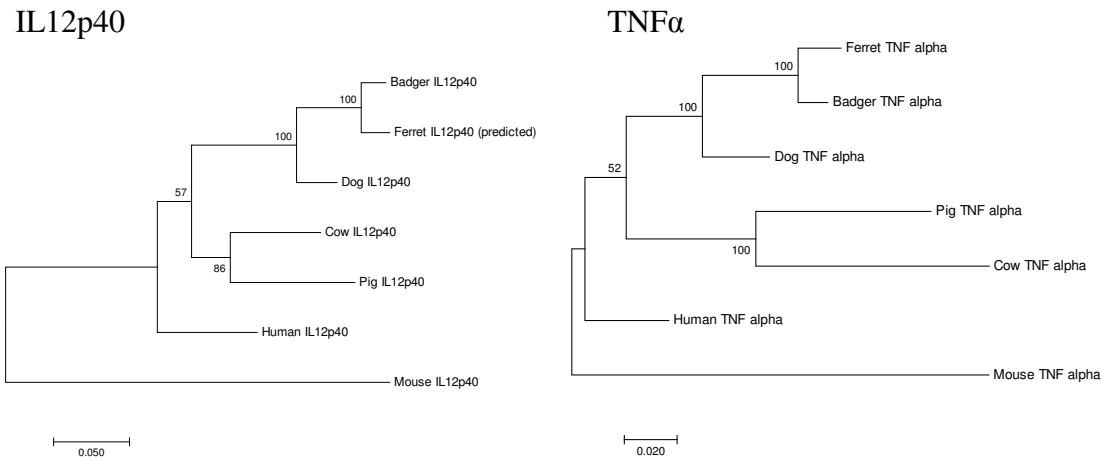
As far as I are aware, these are the first published sequences available for badgers for the genes listed, except for IFNγ, where the sequence I obtained was very similar to that available on NCBI, Genbank, provided by the AHVLA. Levels of identity in amino acid sequences between badger and ferret ranged from 93 to 98%. There was between 63% and 87% identity between badger immune gene sequences (Table 6.4).

Table 6.4 Badger gene homology.

The table shows the percentage amino acid identity between our cloned sequence and ferret/ human seqeunces. Full sequences are indicated in column 2, otherwise location of the partial sequence is provided in identity column.

Gene	Full or partial sequence	Identity to ferret (with amino acid locations)	Identity to humans	Identity to previous badger sequences
IL1β	Full	94%	63%	NA
IL6	Partial	93% (21-114)	65% (3-88)	NA
IL12p40	Full	97%	83%	NA
iNOS	Full	96%	87%	NA
TNFα	Partial	97% (6-196)	87% (6-195)	NA
IFNγ	Partial	98% (26-145)	67% (5-123)	100%
TLR2	Full	95%	76%	NA
TLR9	Full	94%	79%	NA





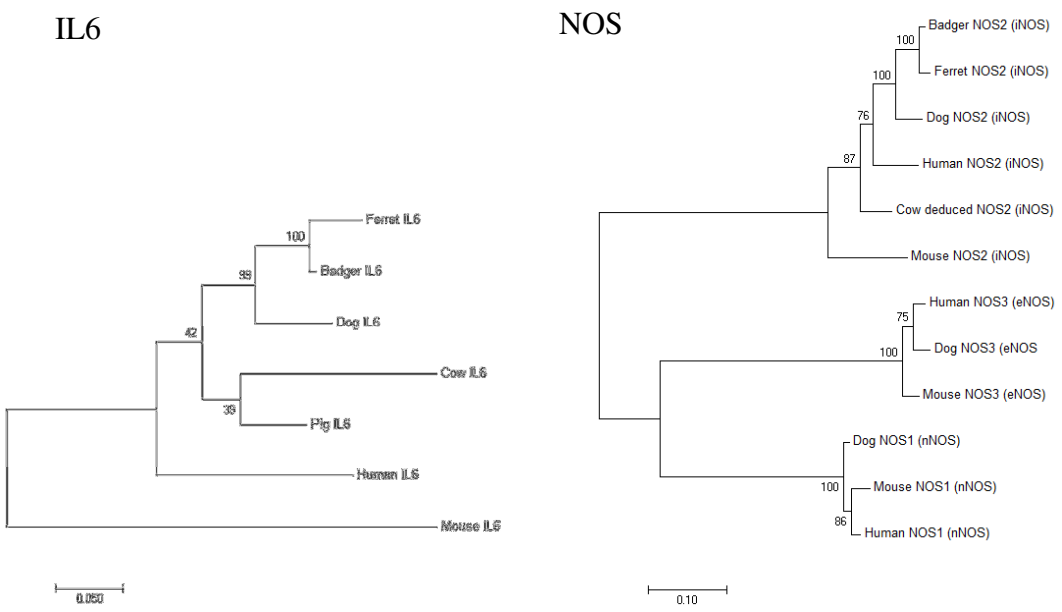


Figure 6.4. Phylogeny of cytokines in mammals

In phylogenetic context, the trees for each cytokine or TLR (Fig. 6.4) are similar to previously established phylogenies for genes in other species (Odbileg *et al.* 2005). The badger genes consistently lie closest to ferret sequences, as would be expected from taxonomic phylogenies. In the phylogenetic trees above, the horizontal distance represents the amount of genetic change (as shown on the scale bar), as the number of changes or substitutions divided by the length of the sequence, while the vertical distance has no significance. The number next to the node indicates how many times bootstrapping (100x) resulted in the same layout as the final tree. The number next to the node thus shows the support for that particular node: The higher the value (out of 100 bootstraps), the stronger the support there is for the node.

6.3.3 Identification of conserved domains and regions in cytokines and TLRs

The alignments (see Supplementary data 1) and domain analyses showed important similarities and conservation of functional domains within many of the badger cytokines compared to closely related species. The major functional domains in IL1β, IL6 and TNFα, as predicted by SMART, are depicted in Figure 6.5.

IL12p40 in badgers and ferrets has an extra domain prediction in the SMART program, compared to the human and mouse predictions (Fig. 6.6A). As well as containing the IGc2 domain present in mouse and human sequences, however, there is an added fibronectin (FN3) domain present. Nevertheless, it appears that this is just an error with the SMART predictor as IL12 in humans and mice also contain an FN3 domain (Yoshiura *et al.* 2003). SMART also does not predict the transmembrane domain present at the start of the badger and ferret sequences.

TLRs are membrane glycoproteins with extracellular domains containing leucine-rich repeat motifs and a cytoplasmic signalling domain (Werling *et al.* 2009). This domain is homologous to the IL1 receptor and termed TIR (Toll/IL1R homology domain). According to the SMART prediction, the badger TLR2 sequence contains two extra Leucine Rich Repeats (LRR) compared to the human and mouse orthologues, and an extra 3 LRRs compared to the ferret sequence (Fig. 6.6). Aside from these differences, badger TLR2 contains the same predicted domains as the orthologues, including a transmembrane region and the TIR domain (Matsushima *et al.* 2007). TLR9 also had differences in predictions for LRR (Fig. 6.7).

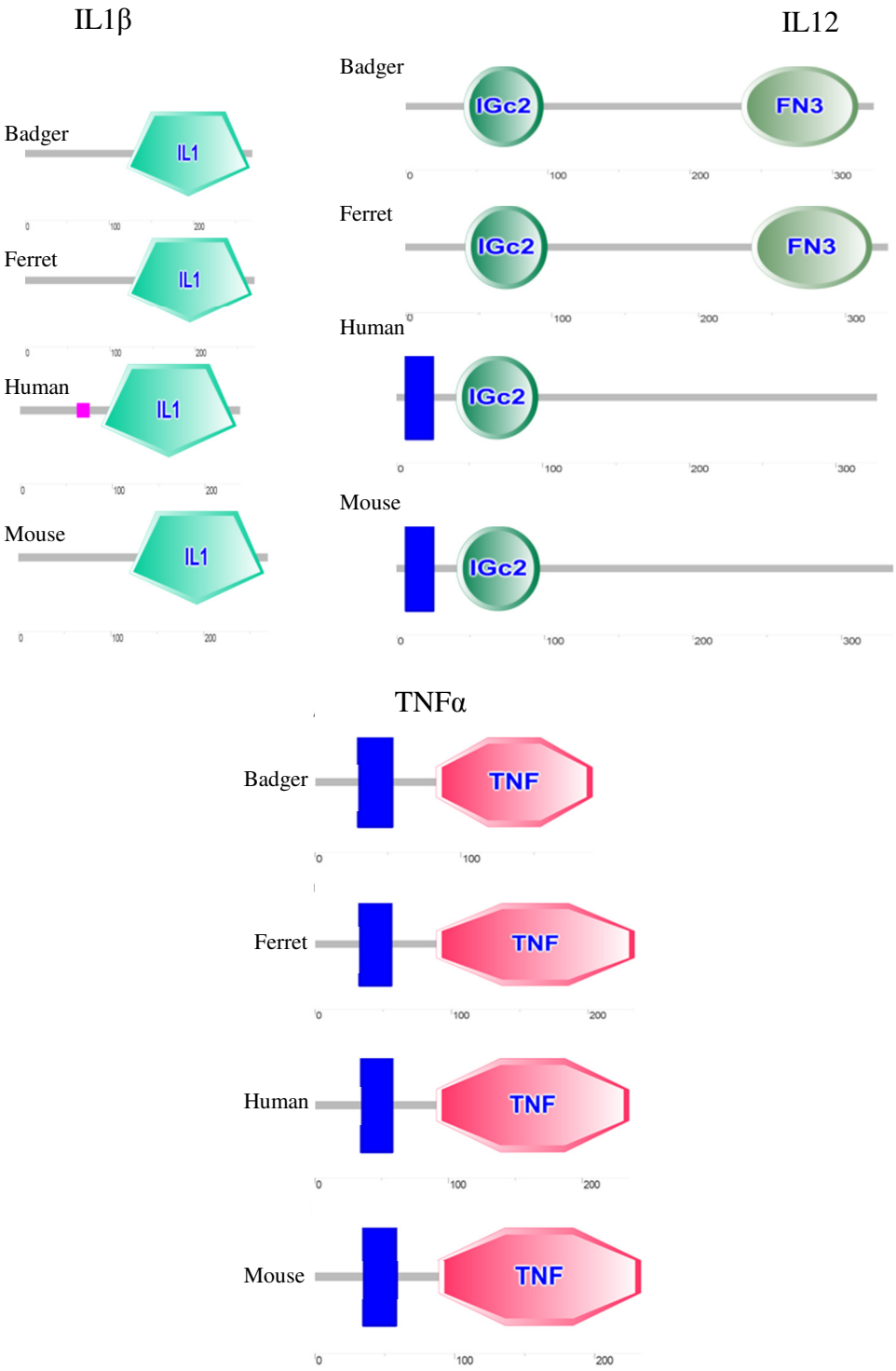


Figure 6.5 The structure of cytokines in badger, ferret, human and mouse, predicted by SMART

Blue rectangles are transmembrane domains, TNF domains are conserved throughout the four species compared, including the newly sequenced badger TNFα.

This observed change in LRRs could have important repercussions in pathogen recognition. In fact the human TLR2 contains 20 LRRs, and TLR9 contains 27 LRRs, but not all of them are detected by the majority of available prediction programs (Matsushima *et al.* 2007). Because TLR 2 and TLR9 are important in the recognition of TB (Kleinnijenhuis *et al.* 2011) and the variability in the number and position of these LRRs could be important in the variability of pathogen recognition (Werling *et al.* 2009), I undertook further detailed manual analysis based on the more detailed human TLR2 and TLR9 annotation (Matsushima et al. 2007).

Twenty LRR or LRR-types domains were identified in the badger TLR2 sequence, along with a transmembrane section and a TIR domain (Fig. 6.6). There are several strongly conserved LRRs between the investigated species, while others are considerably less conserved. Using the structure provided by in Matsushima (2007), I found 27 LRRs and LRR-type domains in TLR9 in the human, ferret, mouse and our badger sequence (Fig. 6.7). In TLR 9, one potentially important amino acid substitution was noted in the badger sequence (denoted by an arrow on Fig 6.7). In LRR9, the 3rd leucine is replaced by a methionine. Because leucines provide so much of the structure in LRRs, this substitution could be important for the recognition of unmethylated CpG motifs (Bell *et al.* 2003, Kędzierski *et al.* 2004).

The take home message from the domain analysis is that it provides useful initial suggestions on conservation and predictions of domains in orthologous genes (Letunic *et al.* 2015), but is not sufficiently detailed to investigate the complex structure of LRRs in TLRs. Badgers have the same overall structure of most genes, but modifications in their LRRs could have resulted in changes to their ability to detect and respond to certain pathogens.

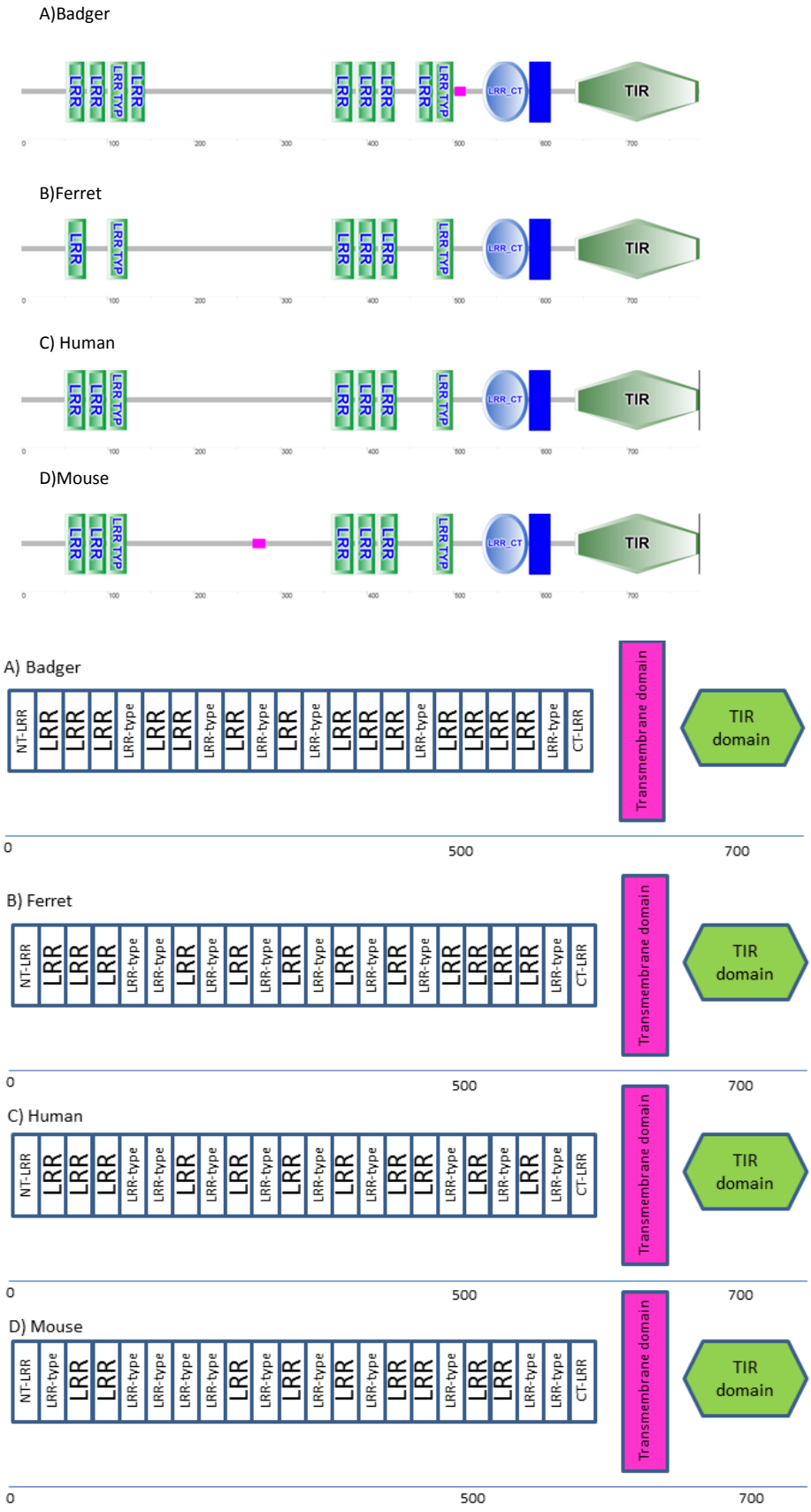


Figure 6.6. TLR2 domain conservation between badger, ferret, human and mouse.

Domains are shown as predicted by SMART (Top) and manual prediction (Bottom).

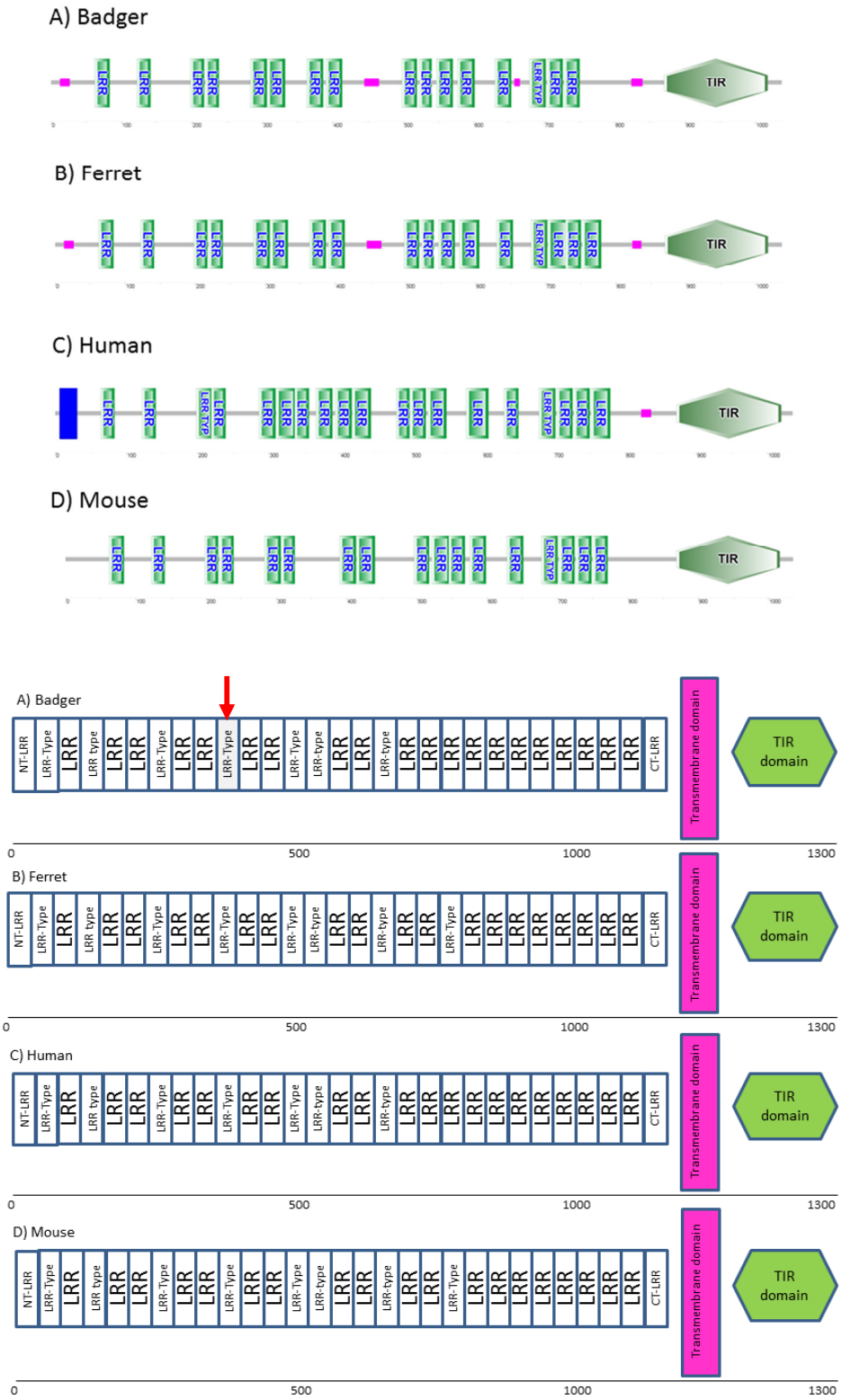


Figure 6.7. TLR9 domain conservation between badger, ferret, human and mouse.

Domains are shown as predicted by SMART (A) and manual prediction (B). The LRR-type which contains a potentially significant mutation for CPG recognition is indicated by the arrow.

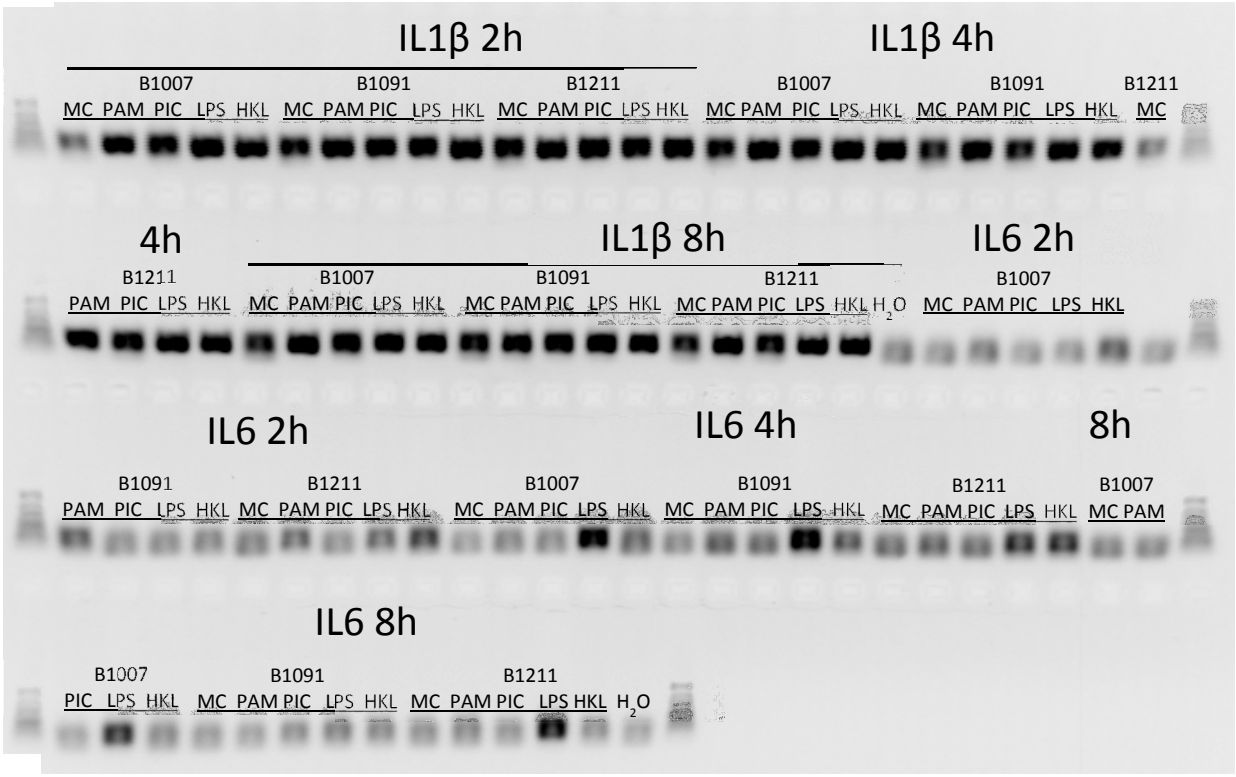
6.3.4 Time-specific upregulation of cytokines, balancing laboratory and field constraints

Ideal laboratory conditions and timings of assays are not always achievable when in combination with field work. Researchers are often required to work in remote settings where the ecological investigation is taking place, away from laboratories. For instance, when investigating cytokine upregulation, eco-immunologists must find a feasible compromise to conduct the experiments within a time frame that fits in with field work. Hence, time course experiments were performed to identify the most informative assay timings for TLR agonist mediated responses in badger macrophages. A time course study was executed with IL1β, IL6, IFNα and TNFα to check at what time points cells start responding to TLR stimulation (Fig. 6.8).

All band sizes were correct based on the designed primers, except for Interferon alpha (IFNα) which had some non-specific binding, probably because of lack of target. IFNα is generally upregulated in response to viral type agonists such as PolyI:C, rather than bacterial agonists (Kawai and Akira 2006). Because this study aimed primarily to investigate innate immune responses to TB, with focus mainly on bacterial agonists, the research and development of IFNα assays were discontinued.

All genes except for IL6 were upregulated at 2 hours post stimulation with a panel of agonists. Stimulation with LPS showed upregulation of IL6 at 4 and 8 hours. For further experiments, where larger numbers of samples were run, it was decided that cells would be harvested 4 hours post stimulation as this seemed to be the best compromise time point to observe all genes, while being short enough for the assay to fit alongside field research schedules.

A.



B.

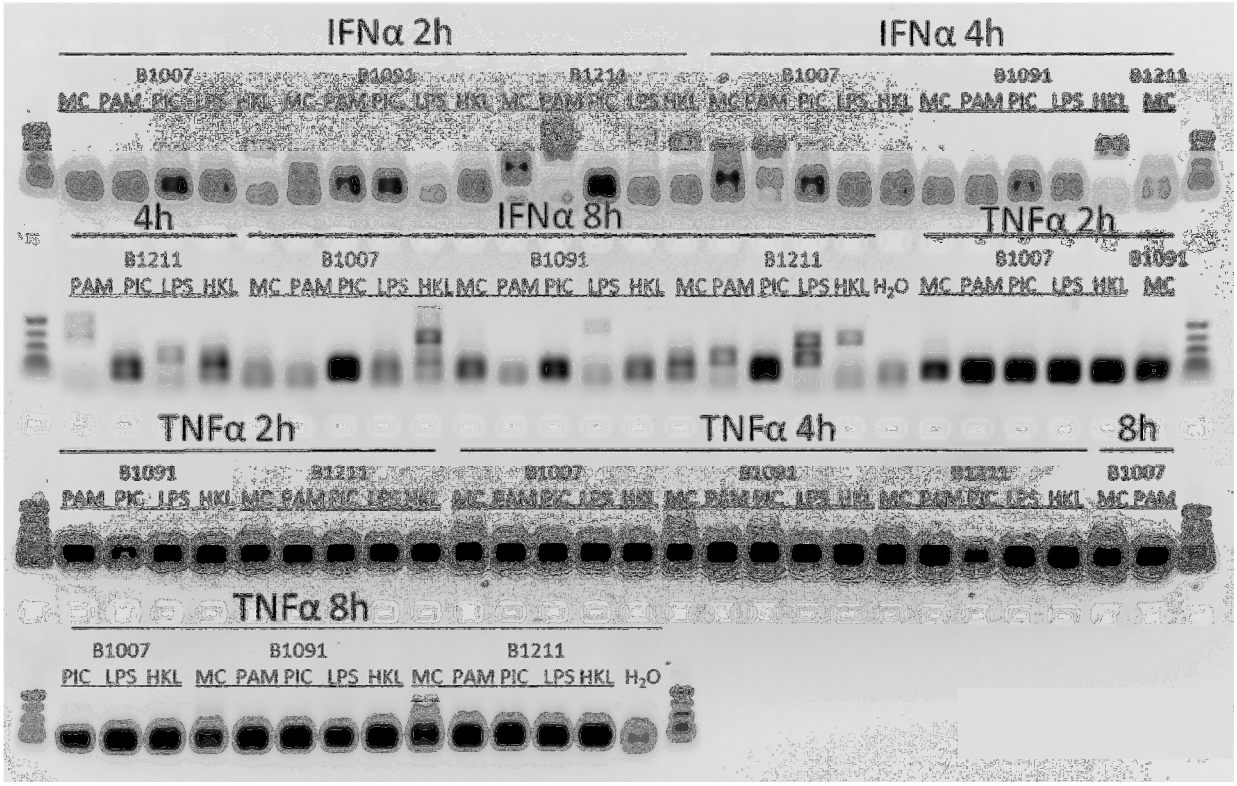


Figure 6.8. Time-course study to evaluate onset of TLR stimulation.

(A) IL1β and IL6, (B) IFNα and TNFα

In summary, when combining field and laboratory assays to investigate immune responses of wild animals, compromises must be made to achieve high levels of results, especially if a single researcher is doing all aspects of the work. Here I show that harvesting cells 4 hours post stimulation was sufficient to see upregulation of cytokines, while not being too time consuming (i.e. no need for overnight assays etc).

6.3.5 **Upregulation of cytokines in response to TLR stimulation**

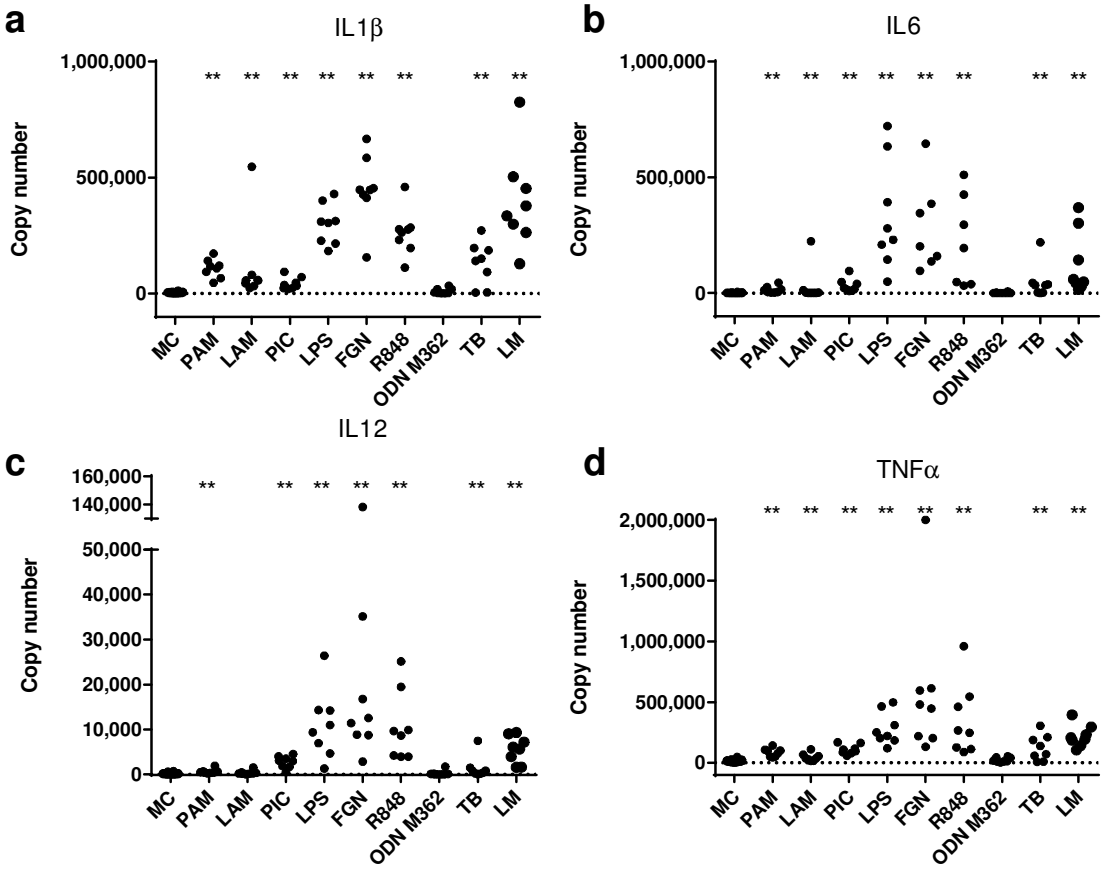


Figure 6.9: Cytokine response to macrophage TLR stimulation

a) IL1β, b) IL6, c) IL12, d) TNFα

A healthy pro-inflammatory response (IL1β, IL6, IL12p40 and TNFα) was observed in response to Flagelin, Imiquimod, LPS and heat-killed *Lysteria*. Responses to PAM, LAM and TB were lower than observed in other species (Redlich *et al.* 2013), with an

almost undetectable response to ODN M362 (Fig. 6.9)(Wagner 2004, Roberts *et al.* 2005). Exposure of bdMφ to heat-killed *M. bovis* BCG induced upregulation of IL1β mRNA to levels comparable with those induced by exposure to heat-killed TB (Figure 6.10).

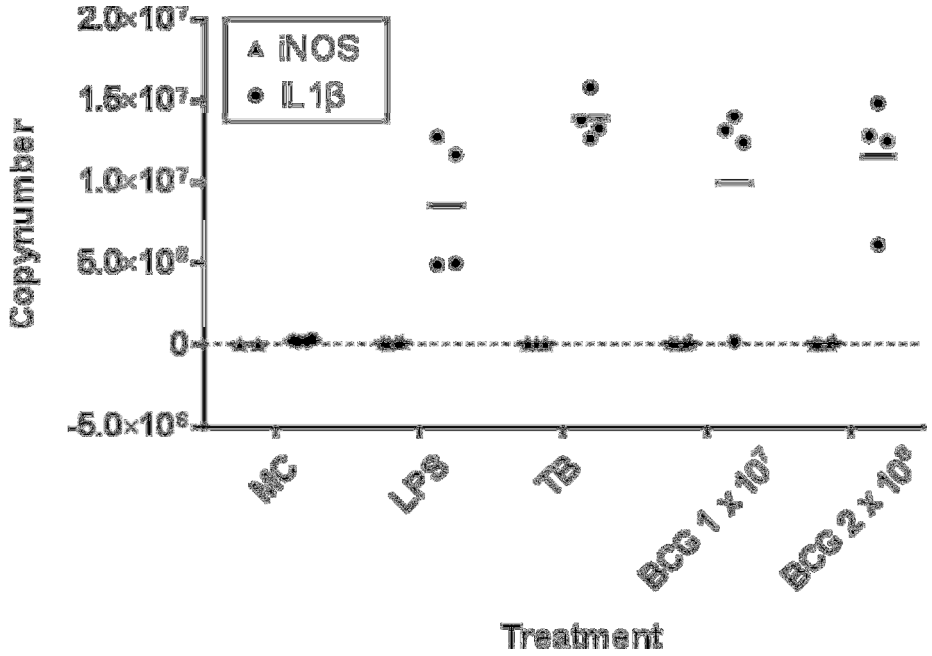


Figure 6.10: Production of cytokines by badger macrophages in response to LPS, TB lysate and heat killed BCG.

Cells were cultured for 48 hours prior to stimulation for 4 hours. MC= media control, LPS= lipopolysaccharide at 1µg/ml, TB= heat killed *M. tuberculosis*, BCG= heat killed *M. bovis* Bacillus Calmette-Guerin

Responses to TLR2 agonists at these concentrations often elicit equivalent cytokine responses to TLR4 agonistd in mice and humans (Takeuchi *et al.* 1999, Varadaradjalou *et al.* 2003), but for badgers there was significantly less upregulation of IL1β to TLR2 agonists than to TLR4 agonists.

Despite the complex agonist mixture in bacterial lysates HKLM and HKTB, which stimulate multiple TLRs to evoke an immune response, the responses varied considerably between individual badgers, especially for heat-killed TB. This could

impact on how badgers respond to bTB and how the disease affects individuals, which will be discussed further in Chapter 7. Across the entire array of different TLR agonists used, no biologically relevant upregulation of iNOS was evident under any circumstance (see Chapter 7 for more detail).

Stimulation of badger TLRs in macrophages showed high levels of pro-inflammatory cytokines, despite some differences with previously established work in yeast, mice and humans. This shows that despite the lack of iNOS production, cells are recognising TLR agonists and microbial lysates and the genes identified in this chapter are transcribed in response to these treatments.

6.3.6 **Bioactivity of IFNγ**

Upregulation of TNFα was observed in response to macrophage stimulation with recombinant IFNγ (Fig. 6.10). This demonstrates the bioactivity of the molecule that was synthesised (Hart *et al.* 1989), although this was still insufficient to upregulate iNOS in badger macrophages (Lu *et al.* 1996, Tate Jr *et al.* 2012). The ramifications of this discovery will be discussed in detail in Chapter 7.

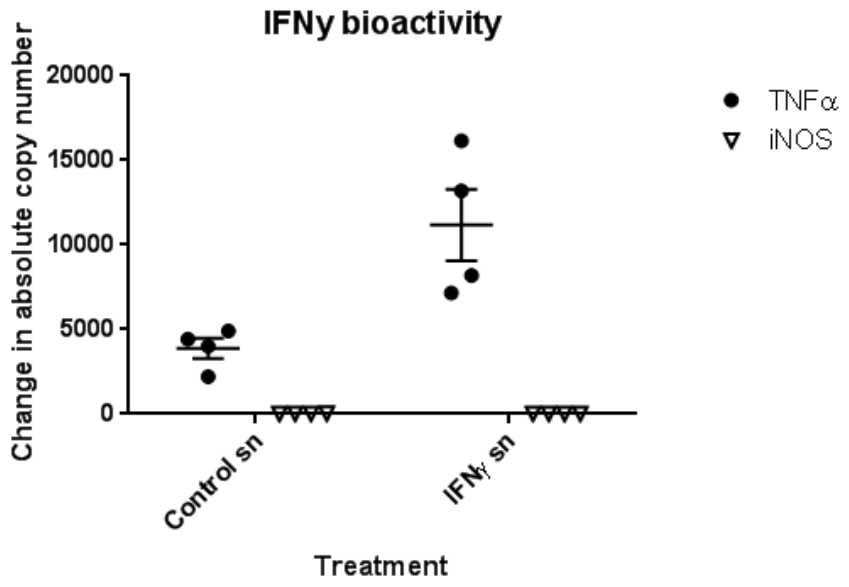


Figure 6.11. Activation of TNFa with recombinant IFNy. No upregulation of iNOS was observed.

6.3.7 **HKTB time course**

Because responses to stimulation with HKTB were lower than those with HKLM in our qRT-PCR data, and bearing in mind I did not use HKTB for the initial time course, a time course for HKTB was completed spanning from 2 to 24 hours post stimulation (Fig. 6.11).

IL1β, IL6, TNFα showed a peak upregulation at 8 hours post stimulation, while IL12p40 showed very little upregulation (maximum at 12 hours for 2 of the 3 badgers). iNOS was not upregulated at any time point. IL12 is a particularly important cytokine for defence against TB (Flynn and Chan 2001a, Hossain and Norazmi 2013). IL12 is mainly involved in the induction of IFNγ expression and the maturation of Th1 cells capable of creating a protective granuloma and is therefore crucial in fighting TB infections (Cooper *et al.* 1997, Cooper *et al.* 2007).

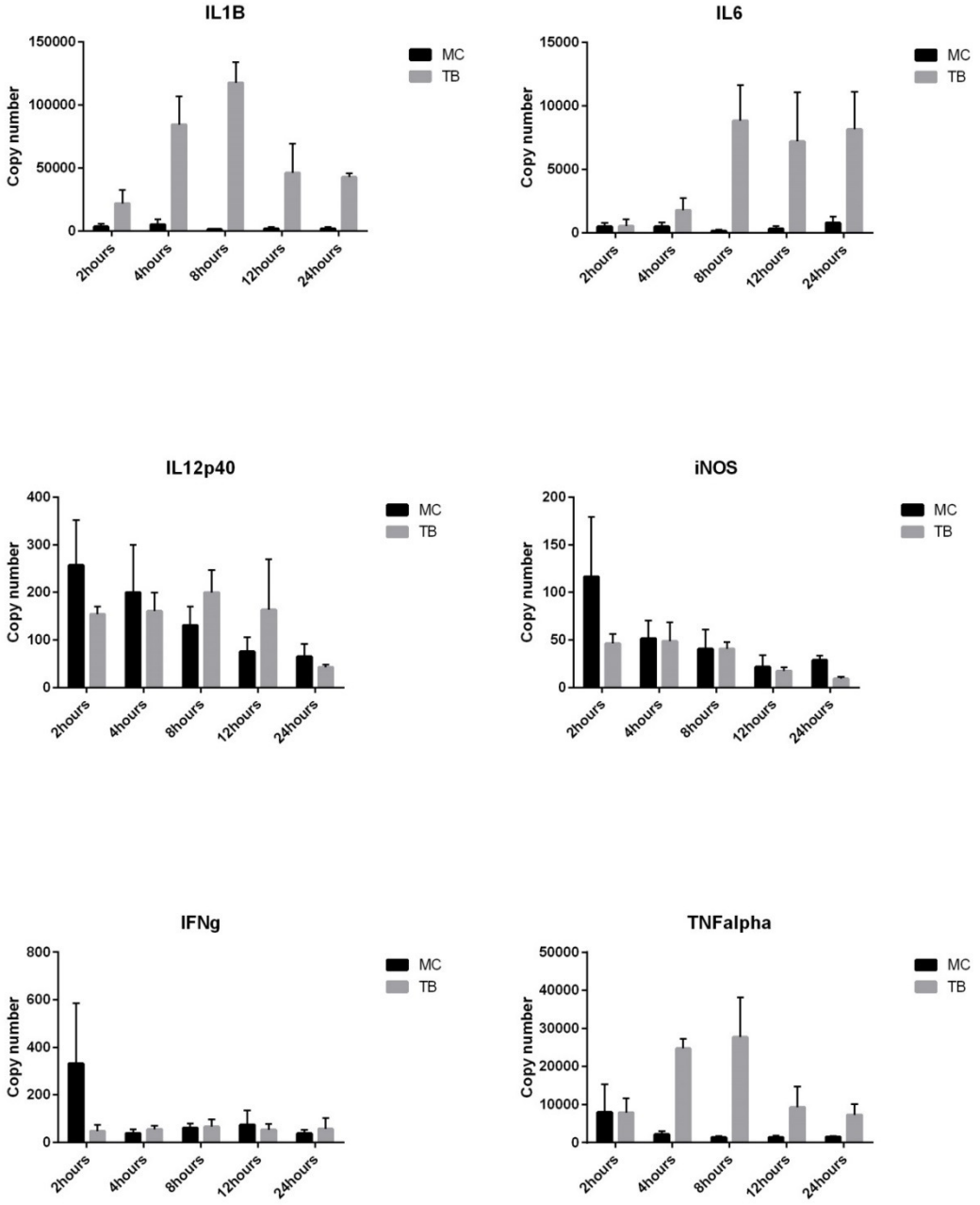


Figure 6.12 Heat-killed TB time course cytokine profiles.

These results show that responses to TB in terms of cytokine mRNA upregulation can be detected within the time frame examined. Nevertheless, this is not true for iNOS. The lack of detectable nitric oxide even after 48 hours stimulation (see Chapter 7) suggests that badger macrophages do not upregulate iNOS under these conditions. The inability of badgers to respond to TB by the production of iNOS, as well as their

decreased IL12 response compared to other agonists could be important in the management of bovine TB in the UK as will be discussed in Chapter 7.

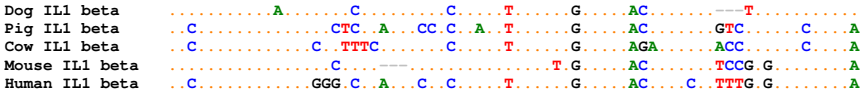
6.4 **Conclusion**

Our findings highlight that there are important, specific benefits arising from investigating wild species directly, rather than extrapolating from common lab analogues and/or gene orthologues. Most studies of innate immunity on model species use inbred rodent strains, which can exclude the intrinsic variability occurring in wild, natural populations. Even for human medicine, studies from mice can be hard to extrapolate because of intrinsic variation in human populations compared to laboratory species (Consortium 2010). Consequently, 'real-world' subjects can inform far more precisely, and thus help in the interpretation of medical research data.

Despite the fact that 22% of mustelids are endangered and carry a variety of pathogens, of which many are epizootic and / or zoonotic (Newman and Byrne in press), very little research has been carried out on wild mustelid immune systems. Ferrets have been used as a model for human flu-vaccines (Maher and Destefano 2004, Buhnerkempe *et al.* 2015), paving the way for more in depth work on other wild mustelids. Indeed many species have been the focus of epidemiological based research such as study of Aleutian Mink Disease in mink (Persson *et al.* 2015) and skunks (Nituch *et al.* 2015), the plague (Williams *et al.* 1994) and canine distemper (Williams *et al.* 1988) in black footed ferrets, as well as the previously mentioned studies of badgers and bovine TB. Despite the economic and conservation importance of these diseases, here I present the first study developing methods for investigation of pathogen recognition receptors in the development of an immune response in a wild mustelid.

6.5 Supplementary data

IL1β

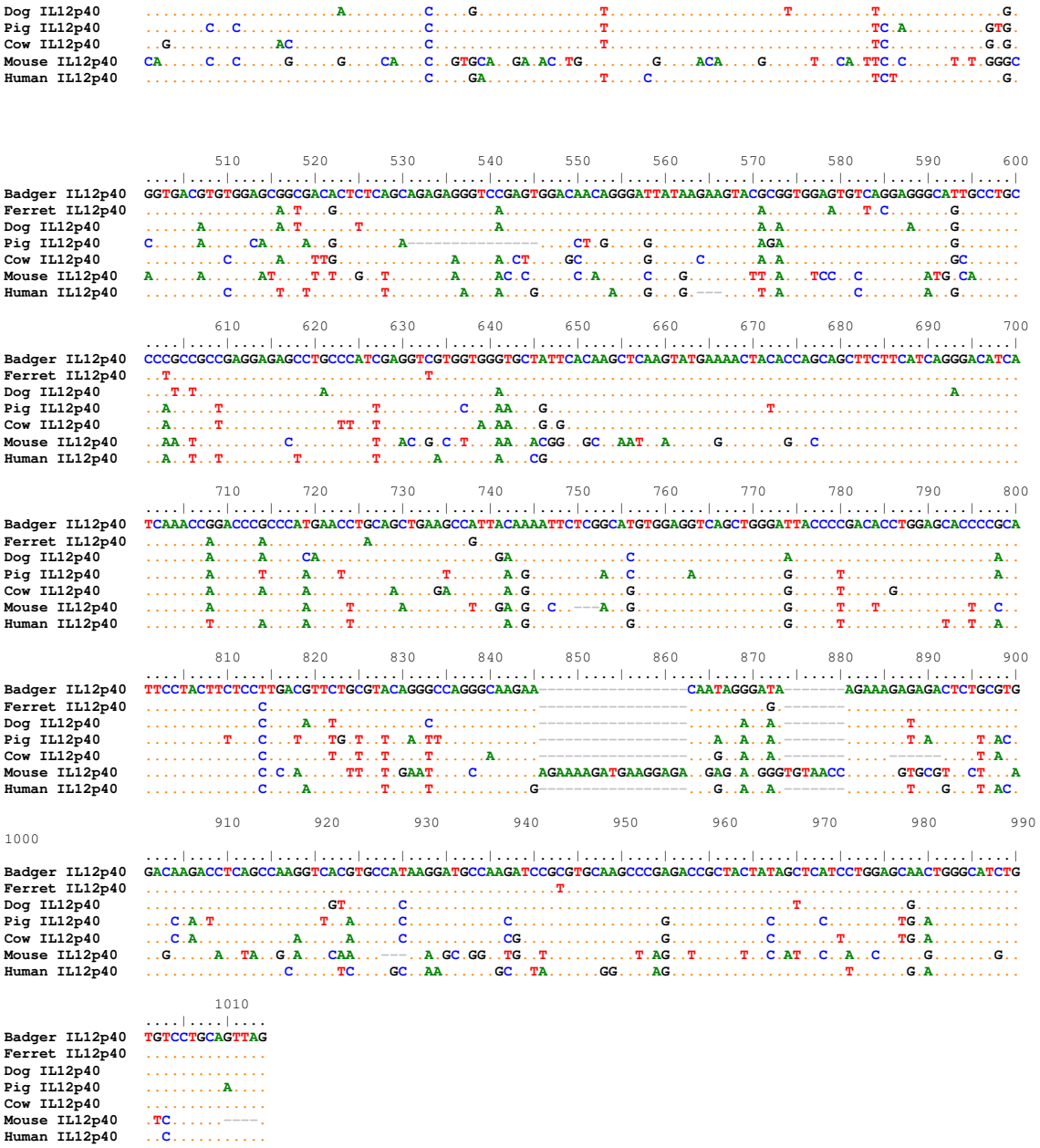

Dog IL1 beta
Pig IL1 beta
Cow IL1 beta
Mouse IL1 beta
Human IL1 beta

IL6

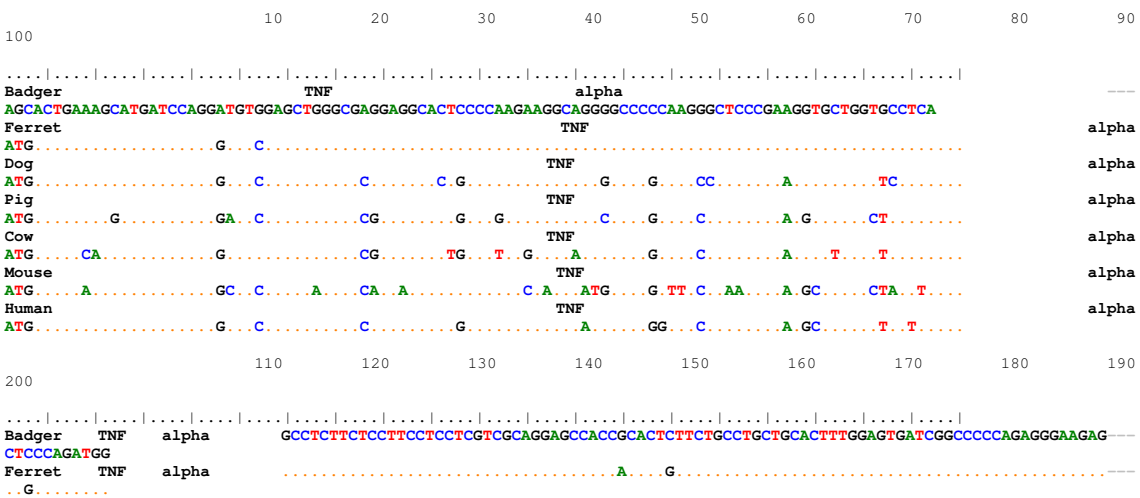

Badger IL6
Ferret IL6
Dog IL6
Pig IL6
Cow IL6
Mouse IL6
Human IL6

IL12p40


Badger IL12p40
Ferret IL12p40
Dog IL12p40
Pig IL12p40
Cow IL12p40
Mouse IL12p40
Human IL12p40


Dog IL12p40
Pig IL12p40
Cow IL12p40
Mouse IL12p40
Human IL12p40
Badger IL12p40
Ferret IL12p40
Dog IL12p40
Pig IL12p40
Cow IL12p40
Mouse IL12p40
Human IL12p40

TNFα


Badger
TNF
alpha
Ferret
TNF
alpha
Dog
TNF
alpha
Pig
TNF
alpha
Cow
TNF
alpha
Mouse
TNF
alpha
Human
TNF
alpha
Badger TNF alpha
Ferret TNF alpha

```
Dog   TNF   alpha            ............................G......A..................................................---
......A....
Pig                                                     TNF                                                      alpha
...................G...............A.G...........A........C.AG..T.............A.......TTT.CAG.T.GCCC
Cow                                                     TNF                                                      alpha
...................G..T............A.G....................C..G..A.....................CAGTC.....G...
Mouse                                                    TNF                                                     alpha
..........A.....G..T..G.....G......A.G........T..A...A....C..G........T.....A.....T...AAGT.....A....
Human   TNF   alpha          ................GA....G.....C......A.G................................................---
T....CAGG.A

                          210        220        230        240        250        260        270        280        290
300

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Badger                 TNF                    alpha                                 CCTCCAACTAATCAAACCTCTGGCCCAG------
ACAGTCAAATCATCTTCTCGAACTCCAAGTGACAAGCCTGTAGCTCATGTTGTAGCAAACCCCGAA
Ferret                 TNF                    alpha                                 ............................------
..............................................................T...
Dog           TNF           alpha                                                   .............GC......T......------
.......................C..............A...........................
Pig      TNF      alpha                                   .T.GAGCA.C.A.---...........AGGA------C...G......G....A...CT..---
..T.....C..C..C..C........C..TGT.A..
Cow           TNF          alpha                                                 ..C.TCCA.C.A..GC.......TT..AACA------
C...GG..C.......A.G.CT.....A.......G.....C..C........CG..AT.A.C
Mouse                                                    TNF                                                     alpha
.....CT..C....GTT..A........ACCCTC...C...G...........A..A.T.G.................C..C..C..........A.C..
Human                  TNF                alpha                                     ....TCT......GC............---G---
G......G...............C..G.................C.................TC..

                          310        320        330        340        350        360        370        380        390
400

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Badger                                                    TNF                                                    alpha
GCTGAGGGACAACTCCAATGGCTGAGCCGGCGTGCCAATGCCCTCCTGGCCAATGGTGTGGAGCTAACAGACAACCAGCTAATAGTGCCATCAGACGGGC
Ferret                                                    TNF                                                    alpha
........G....................A...................................G..................................
Dog                                                      TNF                                                     alpha
........G..G.....G...........A.......................C..C........G..............G........G.....T...T
Pig                                                      TNF                                                     alpha
..C........G..........A...TG..TA.....................C..C...A....G.A............GG.G.....GA....T....
Cow                                                      TNF                                                     alpha
T..CC...G..G....GG...TG.GA.TC.TA.............A.......C......A....GGA............GG.G.....TG.T.......
Mouse                                                     TNF                                                    alpha
.TG....AG..G..GG.G..........A...C.....C..............C..CA....T..C.A.........A...G.G......G.C..T...T
Human                                                     TNF                                                    alpha
........G..G.....G.......A...C..G.......................C........G.G...T........GG.G...........G..C.

                          410        420        430        440        450        460        470        480        490
500

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Badger                                                    TNF                                                    alpha
TGTACCTCATCTACTCCCAAGTCCTCTTCAAGGGCCAAGGATGTTCTTCCACCAATGTGCTCCTCACCCACACCATCAGCCGCTTCGCTGTCTCCTACCA
Ferret                                                    TNF                                                    alpha
.......T........G..G................G...........................T...................................
Dog                                                      TNF                                                     alpha
...................G....................G..CC........C..................................C...........
Pig                                                      TNF                                                     alpha
...................G..........G.........C..CC..........C..TT.......T...............A....C...........
Cow                                                      TNF                                                     alpha
.T..............A..G..........G.........C..CC........CCCT..T.......................A.T..A...........
Mouse           TNF           alpha                                .......TG..........G.T...........A.....C..CC.C---
GA.T.C..................G.......A..T...A....A.....
Human                                                     TNF                                                    alpha
...................G....................C..CC.C......C.............................A....C...........

                          510        520        530        540        550        560        570        580        590
600

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Badger TNF alpha  GACCAAGGTCAACCTCCTCTCTGCCATCAAGAGCCCTTGCCAAAGGGAGACCCCAGAGGGGACTGAGGCCAAGCCCTGGTACGAGCCCATCTACC----
-
Ferret                                                    TNF                                                    alpha
..................G.............................A..............................................TGGGA
Dog                                                      TNF                                                     alpha
...A...........A...............................................C...............................TGGGA
Pig                                                      TNF                                                     alpha
..........................................G...........C......G.C....................A..........TGGGA
Cow                                                      TNF                                                     alpha
.............A....G.......................C...............T..G...................T..A..........AGGGA
Mouse                                                     TNF                                                    alpha
.GAG..A..................G..........C....CC.A...C.....T......G.....CT...A........T........A....TGGGA
Human                                                     TNF                                                    alpha
....................................C.....G..................G...................T...........T.TGGGA

                          610        620        630        640        650        660        670        680        690
700

....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Badger TNF alpha  ---------------------------------------------------------------------------------------------------
-
Ferret                                                    TNF                                                    alpha
GGGGTCTTCCAACTGGAGAAGGGGGATCGACTCAGCGCTGAGATCAACCTGCCTGCCTATCTCGACTTTGCCGAATCCGGGCAGGTCTACTTTGGGATCA
Dog                                                      TNF                                                     alpha
GGGGTCTTCCAACTGGAGAAGGGTGATCGACTCAGCGCTGAGATCAATCTGCCTAACTATCTGGACTTTGCCGAGTCCGGGCAGGTGTACTTTGGAATCA
Pig                                                      TNF                                                     alpha
GGGGTCTTCCAGCTGGAGAAGGATGATCGACTCAGTGCCGAGATCAACCTGCCCGACTATCTGGACTTTGCTGAATCTGGGCAGGTCTATTTTGGGATCA
Cow                                                      TNF                                                     alpha
GGAGTCTTCCAGCTGGAGAAGGGAGATCGCCTCAGTGCTGAGATCAACCTGCCGGACTACCTGGACTATGCCGAGTCTGGGCAGGTCTACTTTGGGATCA
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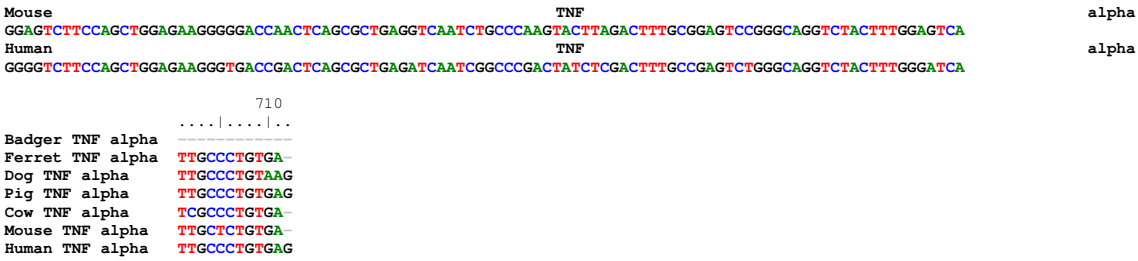
Mouse TNF alpha
GGAGTCTTCCAGCTGGAGAAGGGGGACCAACTCAGCGCTGAGGTCAATCTGCCCAAGTACTTAGACTTTGCGGAGTCCGGGCAGGTCTACTTTGGAGTCA
Human TNF alpha
GGGGTCTTCCAGCTGGAGAAGGGTGACCGACTCAGCGCTGAGATCAATCGGCCCGACTATCTCGACTTTGCCGAGTCTGGGCAGGTCTACTTTGGGATCA
710
Badger TNF alpha
Ferret TNF alpha TTGCCCTGTGA-
Dog TNF alpha TTGCCCTGTAAG
Pig TNF alpha TTGCCCTGTGAG
Cow TNF alpha TCGCCCTGTGA-
Mouse TNF alpha TTGCTCTGTGA-
Human TNF alpha TTGCCCTGTGAG

Chapter 7: **Badger macrophages fail to produce nitric oxide, a key anti-mycobacterial effector molecule**

As submitted to the Journal of Immunology.

Kirstin Bilham, Amy Boyd, Stephen G. Preston, Christina Buesching, Chris Newman, David Macdonald and Adrian L. Smith.

Kirstin Bilham and Amy Boyd contributed equally to the work, and are named as joint first authors on the paper.

7.1 **Abstract**

The European badger is recognised as a significant wildlife reservoir for bovine tuberculosis (bTB); the control of which is complex, costly and controversial. Despite the importance of badgers in bTB and the well-documented role for macrophages as anti-mycobacterial effector cells, badger macrophage (bdMφ) responses remain uncharacterised. I show that bdMφ fail to produce nitric oxide (NO) or upregulate inducible nitric oxide synthase (iNOS) mRNA following treatment with Toll-like receptor (TLR) agonists or recombinant badger interferon gamma (bdIFNγ). Furthermore, the low expression of TLR9 by bdMφ resulted in weak responses to bacterial DNA and a synthetic TLR9 agonist (ODN-M362). Both NO and TLR9 are critical elements of innate immunity to mycobacterial infections, and these features of bdMφ biology would impair their capacity to resist bTB infection. These findings have significant implications for the development of bTB management strategies, and support the use of vaccination to reduce the innate susceptibility of badgers.

7.2 **Introduction**

European badgers (*Meles meles*) are implicated as a major wildlife reservoir for bovine tuberculosis (bTB), the control of which poses substantial practical and political challenges (Delahay *et al.* 2001, Zinsstag *et al.* 2006, Macdonald *et al.* 2015b). In the United Kingdom (UK), attempts to control the spread of bTB cost the taxpayer c. £99m/annum (2013-14; Department of Food and Rural Affairs 2014), yet the problem continues to worsen and control measures have sparked great controversy (Donnelly *et al.* 2006b). Badger culling has been implemented in bTB endemic areas despite strong protests (Donnelly and Woodroffe 2012) yet the efficacy of culling strategies has been questioned (Carter *et al.* 2007, Donnelly and Woodroffe 2015). Vaccination of badgers with Bacillus Calmette–Guérin (BCG) is efficacious under laboratory and field conditions (Chambers *et al.* 2011, Carter *et al.* 2012b) and a current initiative is examining how badger vaccination can control bTB (Wilson *et al.* 2011). Understanding of badger immune responses to bTB challenge is limited despite the potential for this research to inform better bTB control efforts as well as enhancing vaccine development and deployment strategies. Here I present a critical study of badger innate immunity focussing on macrophages, TLR-based pathogen recognition and outcomes of macrophage activation.

Macrophages are the principal target cell for the growth of mycobacteria and key effector cells in immunity. Efficient anti-mycobacterial responses require induction of strong nitric oxide (NO) responses and mice deficient in inducible nitric oxide synthase (iNOS) are highly susceptible to TB (MacMicking *et al.* 1997). Macrophages produce high levels of NO by upregulation of the iNOS gene in response to stimulation by Toll-like receptor (TLR) agonists and/or cytokines such as interferon gamma (IFNγ). Mammals typically express 10 - 12 TLRs that recognise different pathogen-associated

molecular patterns (PAMPs; Akira *et al.* 2006) and initiate a signalling cascade, triggering macrophages to produce cytokines (including IL1β, IL6, IL12 and TNFα) and enter an enhanced antimicrobial state (reviewed in O'Garra *et al.* 2013). In humans, single nucleotide polymorphisms in TLR1, 2 and 9 associate with increased susceptibility to TB (Zhang *et al.* 2013, Schurz *et al.* 2015) and in murine models, TLR2 and TLR9 knockouts exhibit increased levels of TB replication (Bafica *et al.* 2005). I investigated NO production and cytokine mRNA upregulation in bdMφ following activation of TLR and IFNγ pathways revealing two aspects of macrophage function in badgers which contribute to bTB susceptibility.

7.3 Methods

7.3.1 Ex vivo sampling.

Badgers were blood sampled under sedation with ketamine hydrochloride (Zoetis) as part of an on-going study of their socio-ecology at Wytham Woods, Oxford, United Kingdom (Macdonald *et al.* 2015a). All badgers were released at their site of capture after sampling and full recovery. Ferrets were obtained from a recognised supplier and housed in accredited facilities before termination by Schedule 1 methods; all samples were obtained post mortem. All sampling was performed under Animals (Scientific Procedures) Act, 1986 licence and in accordance with guidance from the University of Oxford's Animal Welfare and Ethical Review Board.

7.3.2 Cell isolation and culture.

Blood samples were collected into lithium heparin vacutainers (*BD Biosciences*) and centrifuged (1500 g, 10 min, 4°C) to isolate plasma. Peripheral blood mononuclear cells were isolated following established procedures (Wigley *et al.* 2002). Briefly, cells were isolated using Ficoll (Life Technologies), washed and resuspended in culture medium

comprising phenol red-free Dulbecco's Modified Eagle Medium (Life Technologies) containing 10% FCS (Foetal Calf Serum, Life Technologies), 2 mM Penicillin and Streptomycin (Life Technologies), and 2 mM L-Glutamine (Sigma-Aldrich). Cells were plated at 5 x 10^5 cells/well on 96 well plates, and either mock-treated or treated with defined TLR agonists, microbial lysates (all obtained from Invivogen, concentrations given in Supplementary Information Table S7.4) or recombinant IFNγ (50 ng/ml). For NO assays and IFNγ bioactivity assays, cells were cultured for 48 hours; medium was removed and frozen for Greiss assay (Promega). For TLR agonist induced cytokine analyses, cells were stimulated for 4 hours. Points shown in figures represent replicate wells where cell lines are analysed (Fig. 7.1A, HD11 and RAW), and cells derived from individual badgers in all other cases.

7.3.3 **RNA extraction and cDNA synthesis.**

RNA was extracted from cells frozen in RLT buffer (Qiagen) using either single columns or 96 well format columns (both Qiagen) following the manufacturer's spin protocol. For qRT-PCR, the input RNA amount was normalised for cDNA reactions across a sample set and cDNA was generated (Life Techologies).

7.3.4 **Badger gene sequences**.

Primers were designed based upon regions of target genes conserved in multiple mammalian genomes (Table S7.2). Appropriate primer sets were used to amplify fragments of badger cytokine or TLR genes from PBMC cDNA using Myfi PCR mix (Bioline), according to manufacturer's instructions. Products were cloned into pGEM TEasy (Promega) and sequenced using plasmid targeting (M13) or gene specific primers with BigDye chemistry (Life Techologies), according to manufacturer's instructions. Sequences were analysed using Bioedit (Hall 1999), and badger specific

sequence used to design primers for 5' RACE PCR. 5' RACE cDNA was generated using a SMARTer RACE kit (Clontech) according to manufacturer's instructions. PCR products generated from RACE PCR products were cloned into pGEM TEasy, as described, or into pTarget (Promega) if longer than 1 kb, and sequenced as above. Sequences were analysed using BioEdit and MEGA (Tamura *et al.* 2013) and compared to human, mouse dog and ferret sequences to identify target regions for intron spanning qRT-PCR assays.

7.3.5 **qRT-PCR assays.**

Intron spanning SYBR green qRT-PCR assays were designed using badger gene sequences (Table S7.2). Assays were tested for specificity by melt curve analysis, and cloning and sequencing of amplicons. Validated assays were then used to analyse cytokine cDNA levels in agonist treated PBMC. Dilution series of linearised plasmids containing cytokine or TLR gene sequences were used to calculate copy numbers. Ct values for TLR or cytokine target amplification for each sample were adjusted using the GAPDH Ct value for the same sample, to account for variation in sampling and RNA preparation. The slopes of a plot of Ct against $\log_{10}$ of the plasmid dilution series present on each plate were calculated. The slopes of the respective gene of interest (GOI) and GAPDH dilution series were used to calculate GOI Ct values and adjusted for differences in input total RNA as follows: corrected Ct value = Ct + (Nt – Ct') 3 S/S', where Ct is the GOI Ct value, Nt is the median GAPDH Ct for all samples within an experiment, Ct' is the GAPDH value of the individual sample, S is the GOI slope, and S' is the GAPDH slope (Wu *et al.* 2010). A 10-fold dilution series of linearised plasmid DNA was prepared for each target, starting at 20 million copies. Ct values for the dilution series were calculated as described for samples. Plots of 40 – Ct against copy number of the serial dilution were used to derive sample copy numbers per well

using the following equation: sample copy number = $y*e^{(ab)}$, where "y" is the y intercept, "a" represents the slope of the dilution series and "b" is the corrected 40-Ct of the sample.

7.3.6 **Recombinant IFNγ**

The published sequence (Dalley *et al.* 2004b) for full-length badger IFNγ including Kozak and signal sequences was modified to encode 2 glycine linker residues and a 6 histidine residue tag at the C terminus, and synthesised (Geneart). The sequence was subcloned into pTarget (Invivogen) using standard molecular biology techniques, and HEK293T cells were transiently transfected using TransIT-2020 (Mirus Bio) and cultured in Freestyle protein-free medium (ThermoFisher Scientific). The presence of a protein of the expected molecular weight (~18kDa) in cell culture supernatants was confirmed by Western Blot, using Penta-His antibody (Qiagen), and quantified using a dot blot with the same reagent, by comparison with a polyhistidine-tagged protein of known concentration. It was used to treat cells at 50 ng/ml.

7.3.7 **Statistics**

Statistical analyses were performed in R 3.1.2 (Dalley *et al.* 2004b, R Core Team 2014). Responses to agonist panels (Figs. 7.1B, 4) were analysed using linear mixed effects models with individual badger as a random effect, and the dose response titration of *E. coli* DNA (Fig. 7.6B) was analysed using a linear model, because a badger random effect was not significant. Where necessary, data were transformed in order to meet assumptions of normality and homoscedasticity of residuals. Probabilities were calculated from linear mixed effects models using the Satterthwaite approximation for degrees of freedom (Satterthwaite 1946), as implemented in the lmerTest package (Kuznetsova *et al.* 2015). Where more than one measurement was involved (Figs. 7.1B,

4), a Holm–Bonferroni (Holm 1979) family-wise error rate correction was applied using function p.adjust(). Mixed models were built using the using the lme4 package (Bates *et al.* 2013), and the linear model using function lm(). TLR2 and TLR9 expression levels (Fig. 7.6A) were compared using a two-tailed paired t-test. Nitric oxide production data (Fig. 7.1A) was compared using a two-tailed paired t-test (function t.test()) with a Holm–Bonferroni correction. IFNγ response (Fig. 7.1C) data could not be conformed to assumptions for parametric tests, so distributions were compared using one-tailed Kolmogorov–Smirnov tests (Smirnov 1948), with a Bonferroni correction for multiple comparisons.

7.3.8 **Sequence alignments**

NOS sequences were aligned using Clustal Omega (Sievers *et al.* 2011) and manually refined, with trimming to remove unaligned or poorly aligned stretches at the N and C termini. The sub-alignment of iNOS sequences was formatted for publication using BioEdit 7.2 (Hall 1999) and CorelDRAW X5. Badger iNOS transcript as described in the main text. Other sequence data was drawn from publically available databases (see Supplementary Materials): RefSeq Release 72 (Pruitt *et al.* 2014); Genbank (Benson *et al.* 2013); Ensembl release 82 (Cunningham *et al.* 2015) Dog genome assembly CanFam3.1 and Ferret genome assembly MusPutFur1.0. Mouse and human iNOS transcripts are the forms most closely corresponding to the badger transcript which are present in publically available databases. Each is supported by RNASeq/EST evidence. TIR domains were identified in TLR peptide sequences using SMART (Letunic *et al.* 2015), and aligned using Clustal Omega. TIR domains were aligned in preference to whole-sequence alignments as functional constraints mean that TIR domains can be aligned more reliably than other regions of TLRs (Roach *et al.* 2005). Alignments in

FASTA format are provided as Supplementary Data 1 (NOS) and Supplementary Data 2 (TIR domain).

7.3.9 Phylogenetic trees

MEGA 6.06 (Tamura *et al.* 2013) was used for model selection and maximum likelihood tree construction, with 100 bootstrap replications. For the NOS tree (Fig. 7.2), a JTT+G model (Jones-Taylor-Thornton, with rates among sites gamma distributed) was employed. For the TIR domain tree (Fig. 7.5), an LG+G+I model (Le and Gascuel, with rates among sites gamma distributed, and with invariant sites) was used. In both cases, five discrete gamma categories were used, gaps were partially deleted (95% coverage cutoff), and a moderate branch swap filter was used. Default tree inference options were used. Altering the number of gamma categories or strength branch swap filter, or completely deleting sites with gaps, did not affect the branching of the trees. Trees are supplied in Newick format as Supplementary Data 3 (NOS) and Supplementary Data 4 (TIR domain).

7.4 Results

7.4.1 Badger macrophages do not produce nitric oxide and fail to effectively upregulate iNOS gene expression.

Despite extensive analyses with 33 different individuals NO was not detected in response to a wide range of TLR agonists (Pam3CSK4, PAM; lipoarabinomannan, LAM; poly (I:C), PIC; lipopolysaccharide, LPS; flagellin, FGN; R848; ODN M362) or lysates of TB or *Listeria monocytogenes* (LM) (Fig. 7.1B, Table S7.1). In contrast, similar treatment of murine and chicken macrophages resulted in substantial NO production (Fig. 7.1A). To test whether this lack of NO phenotype was found in other Mustelidae I examined macrophages derived from ferret peripheral blood monocytes

(Fig. 7.1A), or spleen (Table S1). In both cases, ferret Mφ did not produce NO after exposure to LPS, or other TLR agonists (Table S7.1). To identify the badger iNOS gene I designed primers across conserved regions (using human, mouse, dog, ferret and giant panda genomes) and used these to clone and sequence a fragment of the badger iNOS gene. This sequence was used to develop a qRT-PCR, which revealed that the iNOS mRNA signal was very low in both untreated and TLR agonist-treated Mφ (<1000 copies/1.1 million copies of GAPDH) (Fig. 1B). In comparison, TLR agonist treated murine Mφ upregulate iNOS mRNA to levels equivalent to the levels of GAPDH signal (Tajima *et al.* 2012). Hence, the very low iNOS mRNA levels detected with bdMφ are consistent with the total lack of NO response.

IFNγ initiates a TLR-independent pathway of NO production, which enhances LPS-induced NO production (Ding *et al.* 1988). I therefore cloned badger IFNγ (bdIFNγ) and expressed it in HEK293T cells. Exposure of bdMφ to culture medium containing 50 ng/ml bdIFNγ led to upregulation of TNFα mRNA but not iNOS mRNA (Fig. 1C), or release of NO. Similarly, I did not detect NO after exposing bdMφ to a mixture of LPS and bdIFNγ, or to supernatants from Concanavalin A-stimulated badger peripheral blood lymphocytes (in which upregulation of bdIFNγ mRNA was detected by qRT-RTPCR (Table S7.1)). Furthermore, Ficoll-purified leucocytes (a mixed population of lymphocytes, monocytes and other cells) did not produce NO or upregulate iNOS mRNA after stimulation with Concanavalin A for 48 hours (Table S7.1).

7.4.2 **Badgers have an intact iNOS gene but express an unusual mRNA isoform at low levels.**

Using a combination of RTPCR, 5' RACE and genomic sequencing, I identified an in-frame coding sequence for an iNOS isoform with high homology to iNOS transcripts in

other species (Fig. 7.2). Interestingly, the 5' end of the transcript (corresponding to the first exon) was not homologous with the isoform generally considered the canonical 5' iNOS sequence, but has high homology to a variant identified in RNA from human, mouse, dog and cow. Genomic sequencing revealed the potential for a transcript homologous to the canonical iNOS sequence, but this did not amplify using RTPCR or 5' RACE. The predicted protein sequence of the observed transcript was highly conserved to rodent and human iNOS in structurally important areas and in the active site (Fischmann *et al.* 1999; Fig. 7.3).

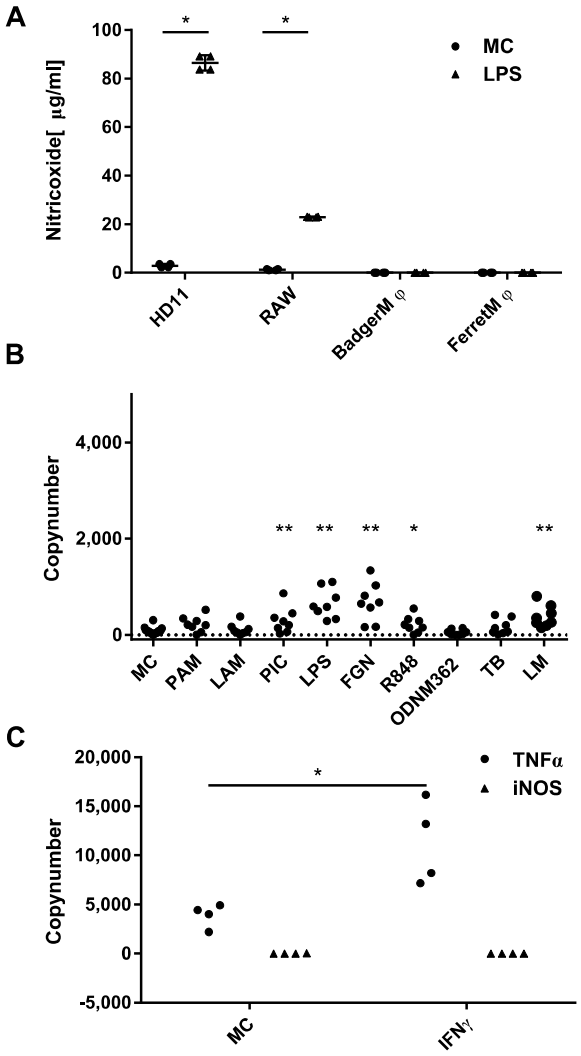

Figure 7.1. Badger macrophages do not produce NO

(A) Badger and ferret peripheral blood macrophages, and mouse and chicken macrophage cell lines, were treated with LPS and supernatants assayed for NO (MC: media control). Error bars indicate standard error of the mean. (B) qRT-PCR was used to measure iNOS RNA in bdMφ following TLR agonist treatment. GAPDH was detected at a mean of 1.1×10^6 copies/well. (C) Badger macrophages were treated with recombinant badger IFNγ and induction of TNFα and iNOS measured by qRT-PCR. qRT-PCR results are given as copy number/well. GAPDH = 1.5×10^5 copies/well. Difference from media control (MC): *=p<0.05, **=p<0.01.

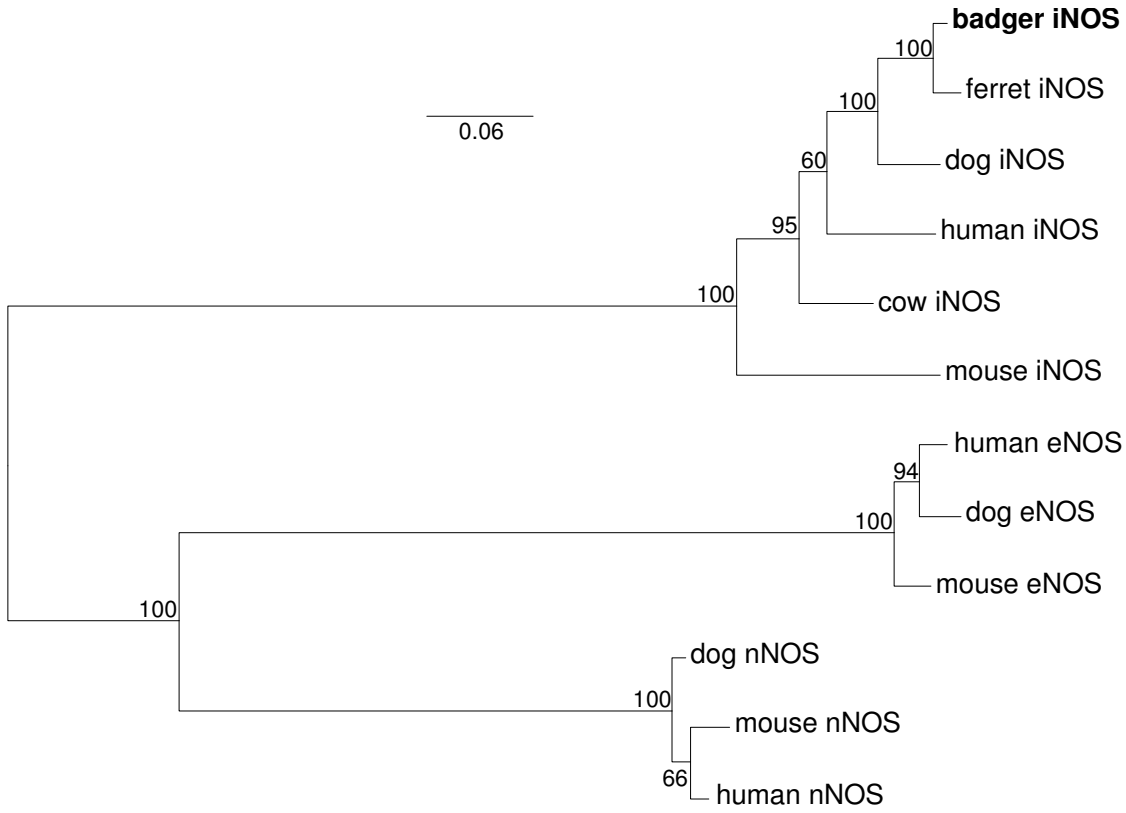

Figure 7.2. The cloned badger NOS transcript is an iNOS orthologue

Translated iNOS (NOS2) sequences were aligned to eNOS (NOS3) and nNOS (NOS1) sequences, manually trimmed to remove poorly aligned ends, and used to build a maximum likelihood tree. Branch labels indicate bootstrap support (out of 100 bootstrap replications).

7.4.3 Badger macrophages upregulate cytokine mRNA in response to TLR agonists and bacterial lysates.

To exclude the possibility that bdMφ were simply failing to respond to TLR stimulation I examined the mRNA levels of various cytokine-encoding genes. Using the cross-species homology-based approach I cloned and developed badger-specific qRT-PCR assays for GAPDH, β-actin, IL1β, TNFα, IL6, IFNγ and IL12 (Table S7.2), employing dilutions of plasmids containing the relevant gene to calculate copy numbers. Agonist-driven cytokine mRNA (IL1β, TNFα, IL6 and IL12) upregulation was evident with a wide range of TLR agonists, as with Mφ derived from other vertebrates (Akira *et al.* 2006).

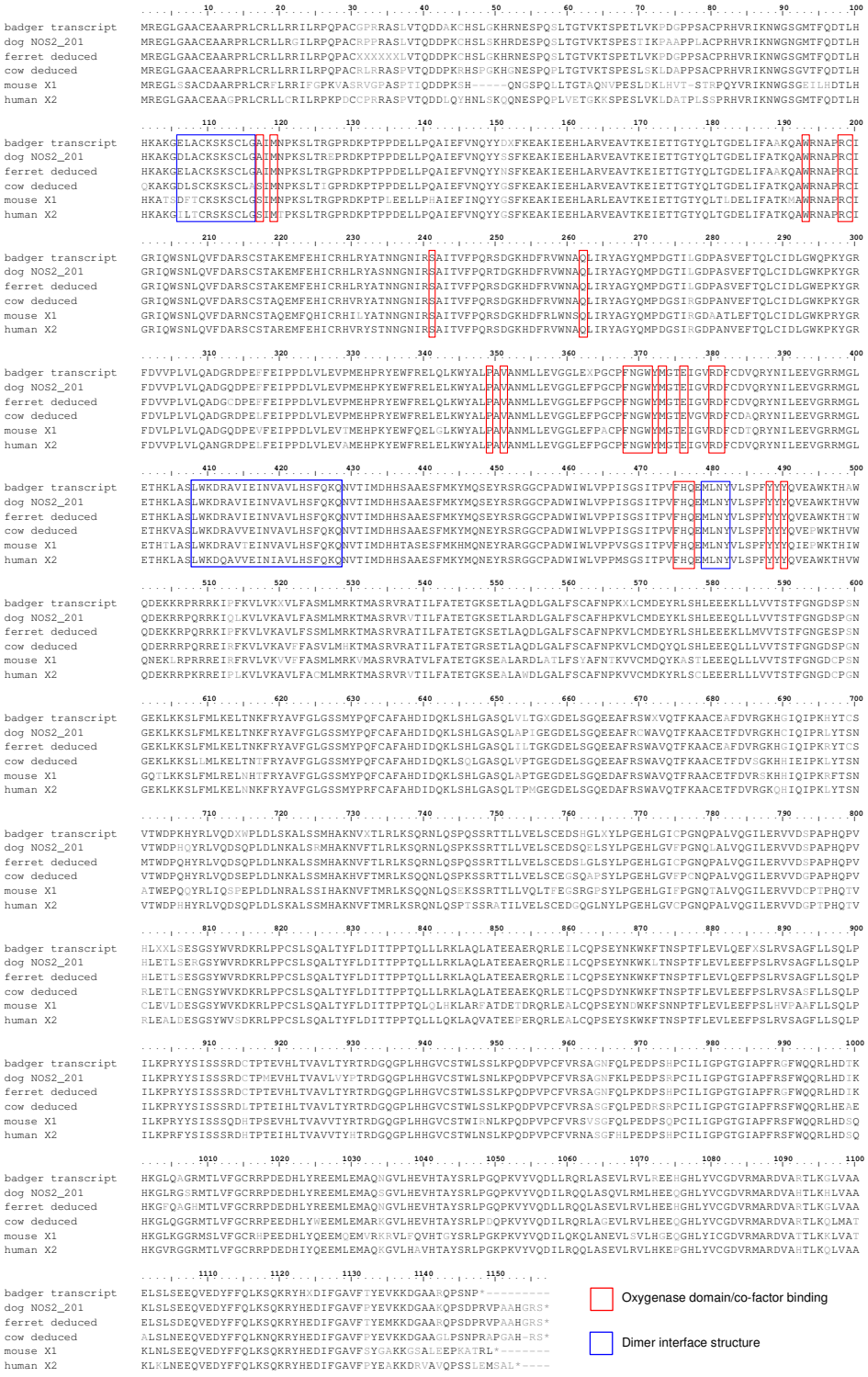

Figure 7.3. Translation of the badger iNOS transcript shows that residues contributing to dimer formation and catalytic activity are well-conserved

The identified badger transcript was translated and aligned to corresponding transcripts, or hypothetical transcripts, from other species. Coloured boxes show sites reported[20] to be involved in dimer formation (blue) or in the oxygenase domain/co-factor binding (red). Residues in black are well conserved (BLOSUM62 score ≥1).

For example, treatment of bdMφ with the TLR4 agonist LPS led to large increases in target gene copy number (mean ± Standard Error for IL1β: 292635 ± 30286; TNFα: 261197 ± 46767; IL6: 331286 ± 83210; IL12: 10827 ± 2762; Fig. 7.4 A-D).The strongest responses occurred with LPS, FGN, R848 (agonists recognised by TLR4, 5 and 7 and/or 8) and heat killed LM. The responses generated to TLR2 agonists, PAM (a synthetic triacylated lipopeptide) and LAM were readily detectable (mean increase in IL1β copy number 102882 ± 13932 and 109799 ± 61799 respectively, although lower than for LPS (292635 ± 30286)), despite using agonist concentrations that induce comparable IL1β responses in other species (Toshchakov *et al.* 2003, Peroval *et al.* 2013). Responses to heat-killed TB preparation were similar to TLR2 agonists. The TLR3 agonist, PIC, caused low but statistically significant increases in TNFα, IL6 and IL12 mRNA. BdMφ did not respond to the TLR9 agonist ODN M362 (a synthetic unmethylated CpG) at 5 μg/ml, which stimulates responses in many vertebrates including humans (Mutwiri *et al.* 2003).

7.4.4 **Badger macrophages express low levels of TLR9 and weakly upregulate cytokine mRNA in response to TLR9 agonists.**

Since TLR9 recognition is important during TB infections (Bafica *et al.* 2005) I considered it important to explore why bdMφ failed to respond effectively to ODN M362. I first sequenced badger TLR9 (and TLR2 for comparison). The sequences obtained for bdTLR9 and bdTLR2 were clear orthologues of these TLR in other species and represented complete open reading frames for both molecules (Fig. 7.5). Secondly,

I compared the level of TLR2 and TLR9 mRNA in bdMφ by qRT-PCR (Fig. 7.6A). Notably the levels of TLR9 mRNA were much lower than detected for TLR2. Thirdly, I exposed bdMφ to a dose titration of *E. coli* DNA (5 - 100 μg/ml) which induced a dose dependent upregulation of IL1β (Fig. 7.6B) although the amounts of DNA required to elicit a response were much higher than required for induction of TLR9 dependent responses in rodents and humans (Bauer *et al.* 2001, Mutwiri *et al.* 2003). NO production was not detected after stimulation of bdMφ with any dose of *E. coli* DNA.

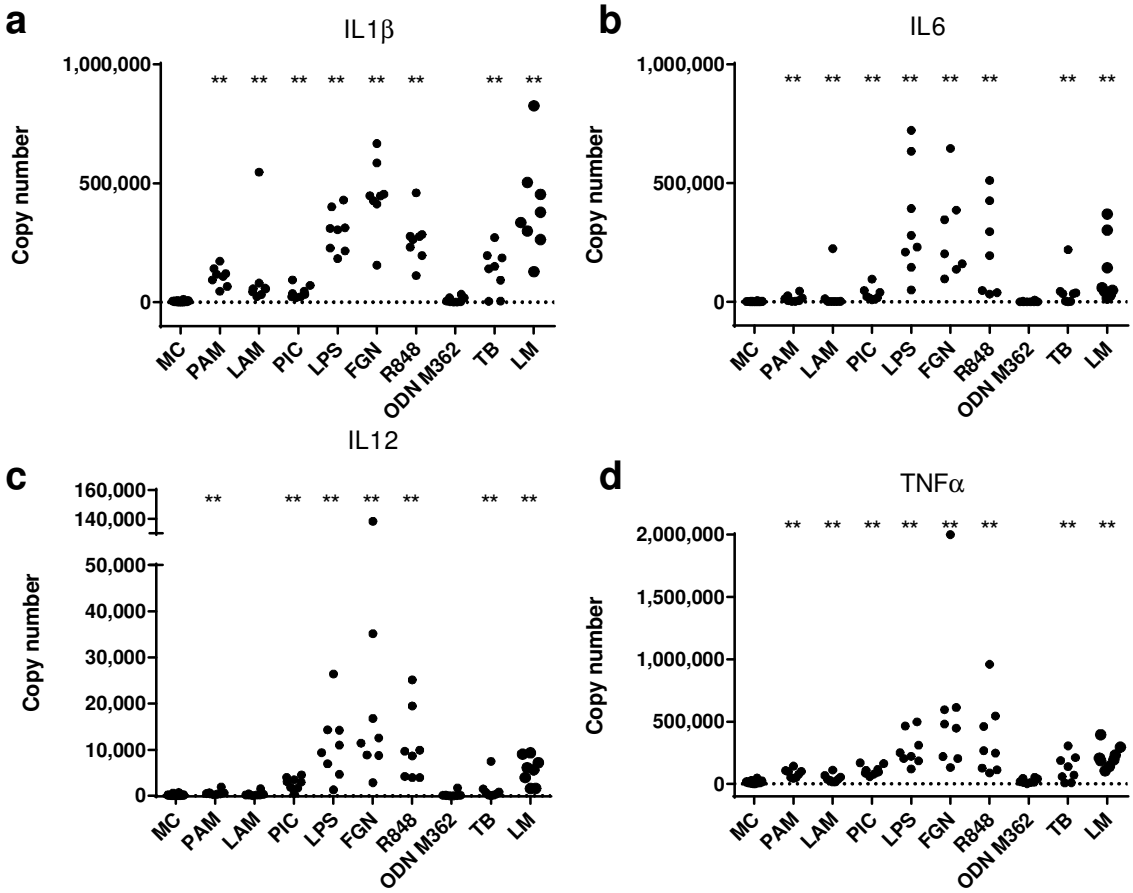

Figure 7.4. Production of cytokines by badger macrophages in response to TLR agonists

qRT-PCR was used to measure cytokine RNA in badger macrophages following TLR agonist treatment (A: IL1β, B: IL6, C: IL12, D: TNFα). qRT-PCR results are given as copy number/well. GAPDH was detected at a mean of 1.1×10^6 copies/well. Difference from media control (MC): *=p<0.05, **=p<0.01.

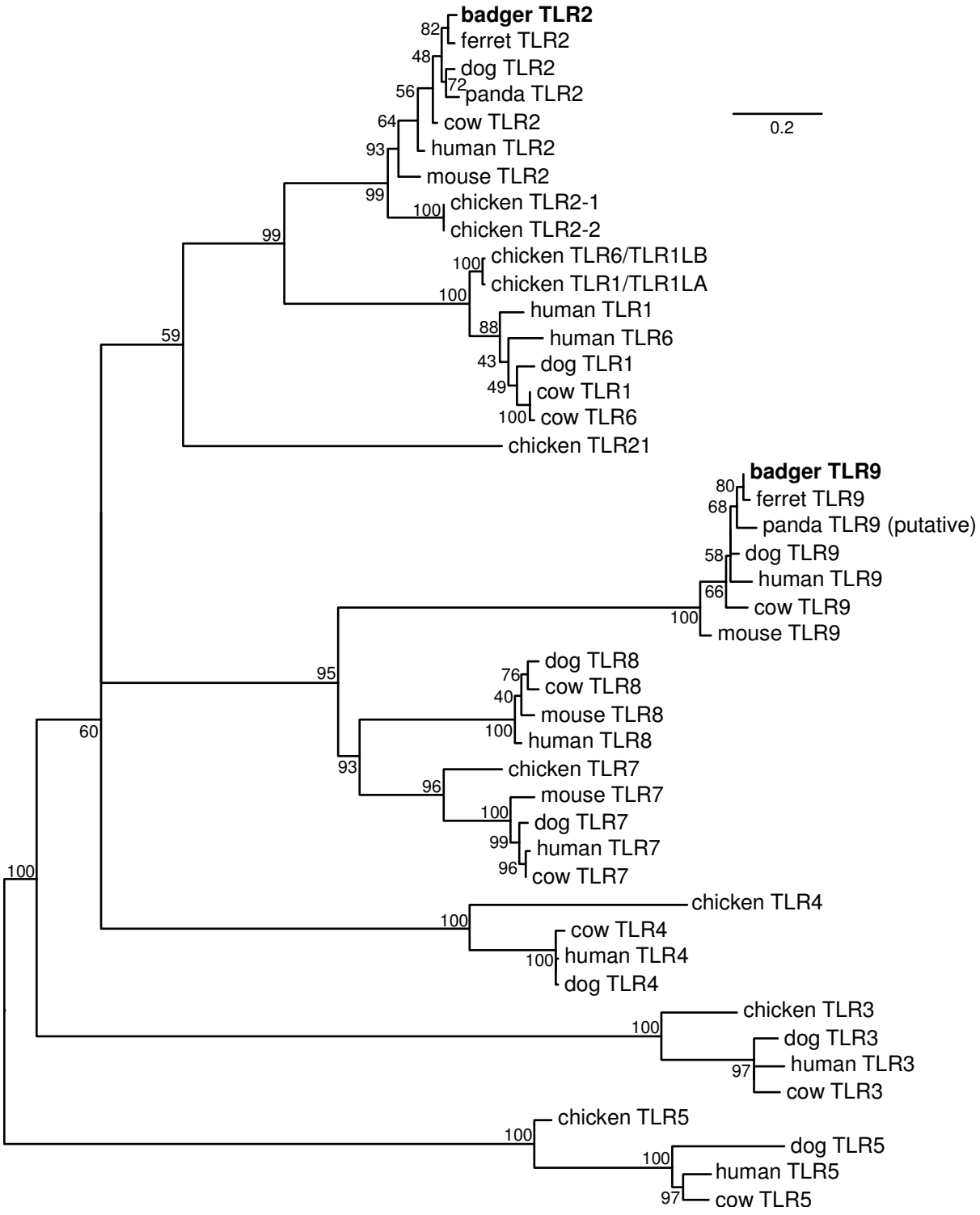

Figure 7.5. The cloned badger TLR2 and TLR9 transcripts cluster with their orthologues from other species

A peptide alignment of badger TLR2 and TLR9 TIR domains with the TIR domains of TLRs from other species was used to build a maximum likelihood tree. Branch labels indicate bootstrap support (out of 100 bootstrap replications).

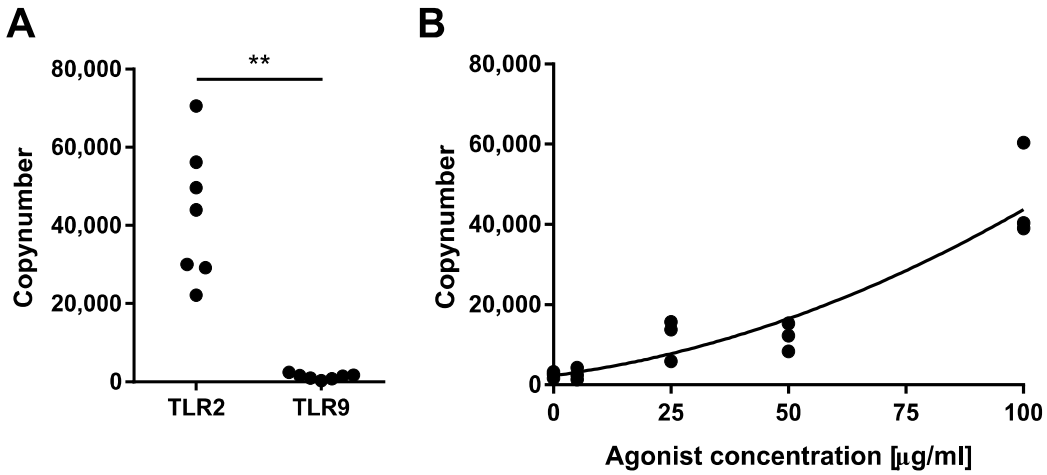

Figure 7.6. Badger macrophage TLR9 expression and response

(A) qRT-PCR was used to measure resting TLR RNA levels in bdMφ. GAPDH was detected at a mean of 3.3×10^5 copies/well. (B) Production of IL1β RNA by badger macrophages stimulated with *E. coli* DNA (effect of *E.coli*, $p < 0.01$, fit line from linear model). GAPDH = 3.1×10^5 copies/well. **=p<0.01.

7.5 **Discussion**

Production of nitric oxide is a feature of the immune response of most vertebrates following treatment with either TLR agonists alone or in combination with cytokines including IFNγ (see references in Table S7.1). Badger macrophages did not produce nitric oxide under any of these conditions. The badger iNOS gene contains an intact coding sequence consequently, lack of iNOS upregulation or NO production after exposing bdMφ to various stimuli could relate to transcriptional regulation, which varies between species (Rao 2000). NO has been implicated as the major anti-bTB killing mechanism with bovine Mφ (Esquivel-Solís *et al.* 2013). Morevoer, iNOS-deficient mice are highly susceptible to TB infection, presenting with atypical granulomas that can facilitate mycobacterial reactivation, dissemination and

transmission (MacMicking *et al.* 1997, Reece *et al.* 2010). The lack of NO production will likely compromise badgers' ability to resist bTB infection and may contribute to the development of atypical granulomas which have a reduced capacity to limit bTB spread.

In addition to the lack of NO, I demonstrate that bdMφ express only low levels of TLR9 and exhibit low responses to TLR9 agonists. TLR9 is involved in bacterial DNA recognition, and is thus important for recognising threats from many different environmental bacteria such as *E. coli*. There is a certain redundancy in immune pathways, as TLR2, TLR4, TLR5 and TLR9 can recognise different elements of various bacteria (REF). As such, the deficiency in one pathway might not completely obliterate the response to bacteria, but might make individuals more susceptible, unless other pathways are able to completely complement / rescue the role of the defective one. Because badgers have solid recognition of bacterial elements through TLRs 2, 4 and 5, this likely explains the capacity to recognise HKTB, and HKLM, despite a lot of variation between individuals. Interestingly, both TLR2 and TLR9 knockout mice have enhanced susceptibility to mycobacterial infections (Bafica *et al.* 2005). Hence, these features of the badger Mφ response may affect the ability of badgers to control infection and provides a functional basis for the badger's innate susceptibility to infection with bTB. Crucially, however, bdMφ can upregulate a full range of cytokines via TLR-dependent pathways, including TNFα, IL12 and IL1β (Flynn *et al.* 1995, Cooper *et al.* 1997, Kleinnijenhuis *et al.* 2011), which contribute to control of bTB infection and lasting immunity. It is thus likely that badgers are using alternative recognition factors than TLR9 to mount an immune response to TB (such as TLR2). Additionally, as other mustelids do not seem to be important reservoirs for bTB, it is possible that if

TLR9/NO deficiencies are common to all mustelids; other factors are involved in the susceptibility of badgers to bTB. Vaccination of badgers against bTB has been shown to be effective under laboratory and field conditions (Chambers *et al.* 2011, Carter *et al.* 2012b). Our study makes two important contributions to this field, firstly, it identifies molecular mechanisms that contribute to the innate susceptibility of badgers to infection with bTB. Secondly, our study identifies TLR pathways that could be exploited to improve the design of adjuvants for future vaccine development. For example, many adjuvents suggested for BCG vaccines include co-stimulation of TLR9. In this chapter I show that this is unlikely to be the best approach, but instead co-stimulation of TLR4 or 5 could help improve the efficacy of the vaccine. In the absence of a suitable vaccine for cattle, vaccination of badgers represents a proven, sustainable and humane approach to the control of this disease in the field. Our study also highlights the importance and potential benefits of studying immune function in wildlife species threatened by zoonotic disease.

7.6 **Acknowledgements**

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Author Contributions: KB led experimental work, designed experiments, collected and processed samples, analysed samples, analysed data and wrote the manuscript. AB led experimental work, designed experiments, processed samples, analysed samples, analysed data and wrote the manuscript. SGP designed experiments, processed samples, analysed samples, analysed data and wrote the manuscript. CDB supervised field work, collected samples and wrote the manuscript. CN supervised field work, collected samples and wrote the manuscript. DWM supervised the ecological study and wrote the manuscript. ALS supervised the immunological and molecular work, designed experiments and wrote the manuscript. Requests for materials should be addressed to: adrian.smith@zoo.ox.ac.uk.

7.7 **Supplementary Information**

Table S7.1. Summary of Nitric Oxide responses in macrophages from different vertebrates

Species	Upregulation of iNOS or production of NO in response to				Reference
	TLR Ag	ConA sup	IFNγ	Mycobacteria	
Badger	No*	No†	No (rIFNγ +/- LPS)	No (lysate)	This study.
Ferret	No	No	ND (not done)	No	[49]
	No*‡	No‡	ND	No (lysate)	This study.
Cow	ND	ND	Yes (rIFNγ + LPS)	ND	[50]
	ND	ND	ND	Yes	[51]
	No	ND	Yes (rIFNγ + LPS)	Yes	[52]
	Yes	ND	ND	Yes	[26]
Human	Yes	ND	ND	ND	[53]
	No	ND	No	ND	[54]
	Yes	ND	Yes (+ LPS)	ND	[55]
	Yes	ND	ND	ND	[56]
	ND	ND	ND	Yes	[57]
Dog	Yes	ND	ND	ND	[58]
Guinea pig	Yes	ND	ND	ND	[53]
	ND	ND	ND	Yes	[59]
Hamster	ND	ND	Yes (+ LPS)	ND	[54]
Mouse	Yes	ND	ND	ND	[53]
	ND	ND	ND	Yes	[12]

	Yes	ND	ND	ND	[60]
Rat	Yes	ND	Yes (+/- LPS)	ND	[54]
Chicken	Yes	ND	ND	ND	[21]
	Yes	ND	ND	ND	[61]

* TLR agonist panel includes Pam3CSK4; lipoarabinomannan; poly (I:C); lipopolysaccharide; flagellin; R848; various CpG motif containing ODN and E. coli DNA.

† Includes bdMφ treated with ConA supernatant from badger blood leukocytes (after 48h of incubation) or mixed leucocyte cultures, after 48 hour incubation.

‡ No NO produced by peripheral blood derived or splenic macrophages.

Table S7.2. qRT-PCR primer sequences (Integrated DNA Technologies)

Target (F:forward, R:reverse)	Sequence 5'-3'
IL12_F	CATTTGGGAACTGGAGAAAA
IL12_ R	ACCGGAGCCTAAGACTTCAC
TNFα_F	CCTGCTGCACTTTGGAGTGATC
TNFα_R	CTATTAGCTGGTTGTCTGTTAGCTCCAC
IL6_F	GAACTCCCTCTCCACAAGCG
IL6_R	GCCAGTGCCTCCTTGCTG
IFNγ_F	GAAGAACTGGAGAGAGGAGAGTGAC
IFNγ_R	CCGCTTACTGCTGCTGCTATTG
IL1β_F	CTGTACCTGTCCTGTGTGATGAAAG
IL1β_R	GTTATGTCCTGACCACCTCTGTTATTTCC
iNOS_F	CCTGTGTTCCACCAGGAGAT
iNOS_R	CTTCTCCGGGGTCTCTTCTT
TLR2_F	CTTTTATTTCCCTGCGGAGTC
TLR2_R	TTCCCCGAGGGATTTGTAAC
TLR9_F	CAGCTGCTGCCTAGTTTGC
TLR9_R	TGCACCAGGAGAGACAAGG

Table S7.3. CpG oligodinucleotides (Invivogen)

ODN	Class	Size	Sequence 5'-3'	Stimulatory for
ODN 1585	A	20 mer	ggGGTCAACGTTGAgggggg	mouse preferred
ODN 2216	A	20 mer	ggGGGACGA:TCGTCgggggg	human/mouse
ODN 2336	A	21 mer	gggGACGAC:GTCGTGgggggg	human/mouse
ODN 1668	B	20 mer	Tccatgacgttcctgatgct	mouse preferred
ODN 1826	B	20 mer	Tccatgacgttcctgacgtt	mouse preferred

ODN 2006	B	24 mer	Tcgtcgttttgtcgttttgtcgtt	human preferred
ODN 2007	B	22 mer	Tcgtcgttgtcgttttgtcgtt	bovine/porcine
ODN BW006	B	23 mer	Tcgacgttcgtcgttcgtcgttc	human/mouse
ODN D-SL01	B	26 mer	tcgcgacgttcgcccgacgttcggta	Multispecies
ODN 2395	C	22 mer	tcgtcgttttcggcgc:gcgccg	human preferred
ODN M362	C	25 mer	tcgtcgtcgttc:gaacgacgttgat	Multispecies
ODN D-SL03	C	29 mer	tcgcgaacgttcgccgcgttcgaacgcgg	Multispecies

Note: Bases shown in capital letters are phosphodiester and those in lower case are phosphorothioate (nuclease resistant). Palindrome sequence is underlined.

Table S7.4. Standard concentrations of TLR agonists (Invivogen)

TLR targeted	TLR agonist	Concentration
TLR 2	Pam3CSK4	300 ng/ml
TLR 2	Lipoarabinomannan	1 µg/ml
TLR 3	Poly (I:C)	12.5 µg/ml
TLR 4	Lipopolysaccharide	1 µg/ml
TLR 5	Flagellin	10 µg/ml
TLR 7	R848/Imiquimod	1 µg/ml
TLR 9	ODN M362	5 µg/ml
TLR 9	*E. coli* DNA	50 µg/ml
Multiple	Heat killed *Mycobacterium tuberculosis*	1 µg/ml
Multiple	Heat killed *Listeria monocytogenes*	10^{7}-^{108} cells/ml

7.8 Supplementary Methods

7.8.1 Sequences

Mouse and human iNOS transcripts are the forms most closely corresponding to the badger transcript which are present in publically available databases. Each is supported by RNASeq/EST evidence. Mouse transcript X1 = RefSeq XM_006532446; human transcript X2 = RefSeq XM_011524860. Dog NOS2_201 is Ensembl transcript ID ENSCAFT00000046006 (automatic prediction from genomic sequence). Ferret iNOS transcript was deduced by ourselves, with Emsembl genomic sequence GL896917.1: 5,007,764-5,007,959 joined to Ensembl automatically predicted transcript

ENSMPUT00000014484. Cow transcript was deduced from a combination of EST data (5' end: GenBank DN741727.1) and iNOS RefSeq sequence NM_001076799.1.

eNOS and nNOS sequences are from RefSeq: mouse eNOS = NM_008713.4; human eNOS = NM_000603.4; dog eNOS = NM_001003158.2; mouse nNOS = NM_008712.3; human nNOS = NM_000620.4; dog nNOS = NM_001195145.1.

Badger TLR sequences are as described in the main text. Other TLR sequences are from RefSeq: human TLR1 = NM_003263.3; human TLR2 = NM_003264.3; human TLR3 = NM_003265.2; human TLR4 = NM_138554.4; human TLR5 = NM_003268.5; human TLR6 = NM_006068.4; human TLR7 = NM_016562.3; human TLR8 = NM_138636.5; human TLR9 = NM_017442.3; mouse TLR2 = NM_011905.3; mouse TLR7 = NM_001290757.1; mouse TLR8 = NM_133212.3; mouse TLR9 = NM_031178.2; dog TLR1 = NM_001146143.1; dog TLR2 = NM_001005264.3; dog TLR4 = NM_001002950.2; dog TLR6 = NP_001041589.1; dog TLR7 = NP_001041589.1 ; dog TLR9 = NM_001002998.1; chicken TLR1/TLR1LA = NM_001007488.4; chicken TLR2-1 = NM_204278.1; chicken TLR2-2 = NM_001161650.1; chicken TLR3 = NM_001011691.3; chicken TLR4 = NP_001025864.1; chicken TLR5 = NP_001019757.1; chicken TLR6/TLR1LB = NP_001075178.3; chicken TLR7 = NM_001011688.2; chicken TLR21 = NM_001030558.1; cow TLR1 = NM_001046504.1; cow TLR2 = NM_174197.2; cow TLR3 = NM_001008664.1; cow TLR4 = NM_174198.6; cow TLR5 = NM_001040501.1; cow TLR6 = NM_001001159.1; cow TLR7 = NM_001033761.1; cow TLR8 = NM_001033937.1; cow TLR9 = NM_183081.1. Or from UniProt[62]: dog TLR3 = E2RIV9-1; dog TLR5 = F1Q0H5-1; dog TLR8 = F1PEB2-1; ferret TLR2 = D7F066; ferret TLR9 = G9KTJ9. Or from predicted transcripts from Ensembl release 82 (giant panda genome assembly ailMel1 / ferret genome assembly MusPutFur1.0):

panda TLR2 = ENSAMET00000020274; panda TLR9 (putative, identified by BLAT search) = ENSAMET00000009973; ferret TLR2 = ENSMPUT00000019046; ferret TLR9 = ENSMPUT00000011807.

Chapter 8: **Synthesis: The importance of cellular physiology and molecular immunology in evolutionary ecology and applied conservation**

It is paradoxical that the most fundamental process enabling aerobic life is also one of the most detrimental to the body (Dowling and Simmons 2009). Respiration - the metabolic reactions that produce energy through oxidative phosphorylation - is what has allowed eukaryotes to populate a planet so rich in oxygen (Knoll 1992, Aguirre *et al.* 2005). Cellular respiration is, however, also the main producer of reactive oxygen species (ROS), which can be damaging to tissues and ultimately organisms (Bergamini *et al.* 2004). These damaging physiological processes are fundamental to mechanistic explanations for diverse selective traits linked to health, survival and fitness in wild animals (Monaghan *et al.* 2009). Traditional population ecology can identify the age- and sex-specific mortality schedules, and the reproductive profiles of wild-living organisms. It can also ascertain how these are associated with environmental factors, such as prevailing weather conditions, disease or habitat change. What is lacking is a rigorous understanding of the physiological mechanisms that under-score these observations. This requires an improved awareness of the cellular currencies with which organisms interact with their environment (*sensu* McGraw *et al.* 2010). There is currently much debate over the importance of oxidative stress in the ageing process (Finkel and Holbrook 2000, Hekimi *et al.* 2011), where free-ranging organisms are engaged in a molecular battle with free-radicals and other reactive chemical species. Although some reactive molecules provide key functions in the body (e.g. cell signalling and immune function), these same molecules can accumulate to damage

cells, and tissues, making them double edged swords (Martin and Barrett 2002). Ability to resist this oxidative stress and any resultant oxidative damage is key to survival, yet the risks posed by ROS vary with life-history stage, with young, reproducing and elderly individuals most vulnerable (Alonso-Alvarez *et al.* 2006, Selman *et al.* 2012) – where interactions are further influenced by the relative severity of seasonal and annual conditions (Zera and Harshman 2001).

Molecular genetics, the advent of cheaper gene sequencing, development of microarrays and other molecular techniques has seen ecological studies move forward with leaps and bounds over the last 30 years (Lowe *et al.* 2009). A similar paradigm-shift is now taking place in eco-physiology, using physiological markers as indicators of health in wild animal populations (Reeder and Kramer 2005). In this context, the measurement of markers of oxidative balance, such as antioxidant defences and oxidative damage, may become a valuable conservation tool (Reeder and Kramer 2005). These techniques do not replace 'conventional' ecology and conservation techniques, studying population dynamics, behaviour, reproduction and survival, but rather aim to provide complementary mechanistic explanations for variation in these traits (Costantini *et al.* 2010, Beaulieu *et al.* 2013, Chave 2013) through an interdisciplinary and integrated process (Isaksson *et al.* 2011a, Chave 2013).

Despite the value of applying OS to population ecology, many authors and reviews still highlight the variations in methods used, as well as the problems associated with measuring the correct physiological processes given the study questions (Horak and Cohen 2010). Most methods for measuring oxidative stress and antioxidant levels used in ecology have been developed for biomedical studies, which have different constraints

than ecological ones. While there is some useful carry-over (see Romero 2004), techniques often need validation or modification in order to be used in ecology (Beaulieu 2014). Additionally, many of the studies on oxidative stress (OS) ecology have been done in birds (Alonso-Alvarez *et al.* 2007a, Catoni *et al.* 2008), where physiology is inherently different to mammals (Nussey *et al.* 2008, Nussey *et al.* 2009). In this thesis, I advanced understanding of how the OS profiles of a European badger population change with age, with special attention to trade-offs between growth and AOX during juvenile development, and any decline in AOX (increase in OS/OD) with senescence (Chapter 4). I then examined how these traits were influenced by the severity of weather, especially in terms of causing trade-offs, or driving selective mortality in ways that progressively improve the capacity of surviving sub-cohorts to withstand OS (Chapter 5). Linked to this, I also aimed to investigate reactive nitrogen species (specifically nitric oxide - NO) and its role in innate immunity. When it became clear that badger macrophages did not produce NO, I investigated the innate immune system further, with particular regard to bovine tuberculosis; a key disease affecting badgers.

In order to investigate OS, it was first necessary to validate OS techniques to be used in further studies on this species. In Chapter 3, I demonstrated that techniques that suit one species, or ecological question, do not necessarily make it applicable to all other species. For example, correction for uric acid is necessary when making certain measures of antioxidant in birds, but is not required when working with mammals, which have an additional enzyme for uric acid degradation not present in birds (thus birds, and indeed humans – an exception to the mammal rule, accumulate more uric acid than do mammals, Varela-Echavarría *et al.* 1988). Another difference is that,

unlike birds, mammalian red blood cells are not nucleated (Gaehtgens *et al.* 1981), making the study of DNA damage more difficult where blood is the only tissue available; in this thesis I use resistance of RBC cell walls to asses resistance of cells to OS.

Whether juvenile development is a period of high OS has been debated, Metcalfe and Alonso-Alvarez (2010) propose that if ROS production was always high in juveniles, this should be a maladaptive trait which would be phased out by evolution, through increased antioxidant defences, but that this adaptation might be constrained by resource limitations, given the cost of antioxidant defences (Khan and Black 2003). In Chapter 4, I found that badger cubs exhibit higher levels of oxidative damage than adults, and that survival to maturity (age one year) was associated with individuals expressing lower markers of oxidative damage. Such selective mortality where only the strongest cubs survive to maturity (Searcy and Sponaugle 2001, Chen and Maklakov 2012) means that the cohort as a whole increases in "quality" over time. Some measurements of size (foot length) as adults were inversely related to former cub antioxidant capacity within individuals, suggesting trade-offs; note, however, sample size was a constraint on analyses. Thus survival probability is constrained by oxidative damage but somatic growth might be constrained by antioxidant capacity.

ROS production is – to some extent – genetically determined (although infection can induce spikes of ROS / Reactive nitrogen species (RNS) production by the immune system), while antioxidant capacity is related more to environmental conditions (Olsson *et al.* 2009). Thus, some genotypes might be adaptive in certain environments, while

others would be detrimental. Risk of OS can also be linked to habituation, however, as shown by studies in exercise in humans (Niess *et al.* 1998).

Here I give the first indication that high levels of oxidative damage can compromise juvenile survival in a wild mammal, not just future reproduction or adult survival (Bize *et al.* 2008). Previous work on European badger population dynamics has shown that cubs are more susceptible to climate variability and parasite infection than adults (Anwar *et al.* 2000), and that some of this susceptibility has a genetic component (Annavi *et al.* 2014). In Chapter 4 I show that while cubs initially need to be fat enough to survive the first few months, oxidative damage then an important factor in determining survival from summer until adulthood. I also demonstrate that levels of oxidative damage and antioxidant defences relate strongly to environmental conditions. From my results, I propose that genotypes that produce higher levels of ROS could survive longer under conditions allowing strong antioxidant defences, compared to conditions causing low antioxidant defences. While I do not yet have the genetic data available for these cohorts, it is possible that heterozygosity is linked to ability to resist oxidative stress, thus modulating survival through interactions with environmental conditions (Sohal and Weindruch 1996). Sample sizes precluded the separation of survival into years, as well as physiological state, but interactions between these variables would be crucial for a more integrated approach. Figure 8.1 summarises how these factors are linked to survival probability of badger cubs, as well as highlighting additional important missing that will need to be investigated by future research.

Resource availability, based on seasonal changes and weather condition can have impacts on the antioxidant defences, as well as the frequency and duration of foraging

(locomotion/activity) required to survive, but linked to ROS production through metabolic rate (Noonan *et al.* 2014).

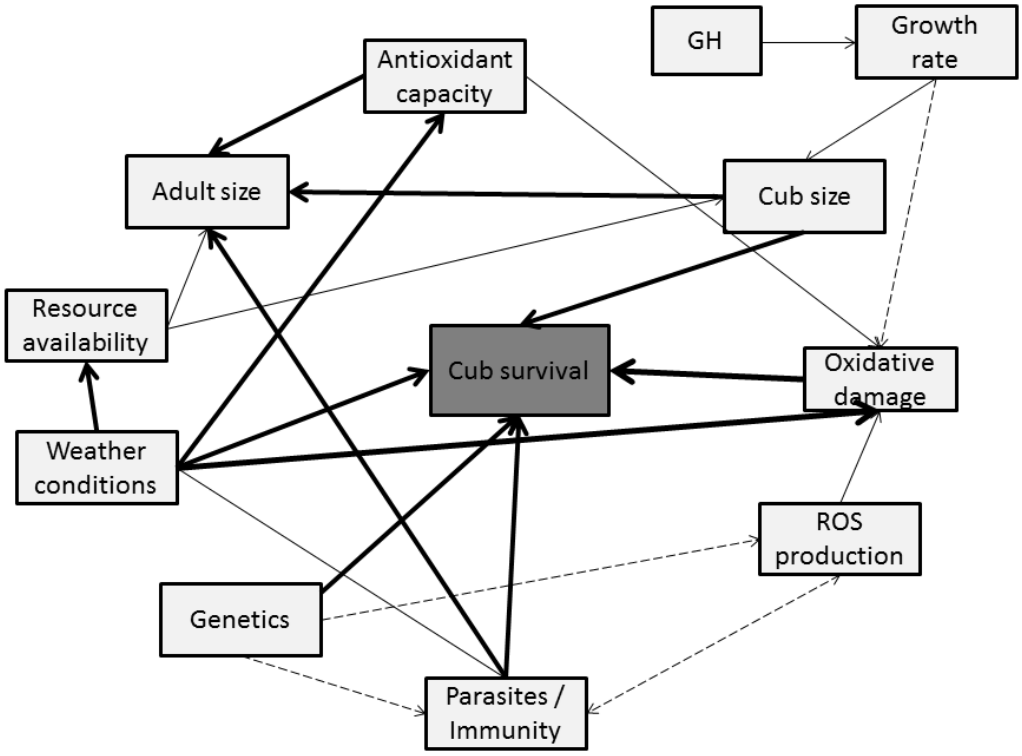

Figure 8.1 Diagram of physiological and environmental interactions affecting badger cubs.

Thick lines correspond to current data available for badgers. Thin lines are elements assumed from established data. Dotted lines are interactions that warrant further attention in badgers, given the other interactions presented and data on other species. (GH = growth hormone)

In Chapter 5 I discovered that carry-over effects (COE: *sensu* Harrison *et al.* 2011) from winter conditions occur in badgers, and that this not only manifests itself in body condition, but also in terms of antioxidant capacity and probably micronutrient stores. I propose that individual "quality" in the spring is determined metabolic balance over the preceding the winter, as the ratio to active foraging to reliance upon fat reserves during

periods of torpor. This means that oxidative condition can be used in combination with body-condition scores or body condition indices in order to evaluate quality of individuals, and their likelihood to reproduce and survive (see Bize *et al.* 2008), with importance for conservation management (Beaulieu *et al.* 2013). For example, knowing what weather factors affect OS and reproduction in a known species would help predict the stressors which a species could be put under, and what management strategies could be put in place. If temperature is a particular driver of OS throughout the winter, refuges could be put in place to minimise these effects. COE of winter were observed an opportunistic omnivore, exploiting a wide variety of food sources (Macdonald *et al.* 2015a). For more specialist species, or trophic systems, where the availability of specific food types is even more heavily determined by seasonal weather conditions (e.g., Zhou *et al.* 2013) physiological quality will be even more strongly affected. In particular in marine species, where avoidance of inclement temperatures is impossible, it would be expected that temperature driven OS could lead to increased mortality and decreased fertility (Lesser 2006, Pörtner 2001). Interestingly, in the coldest winter examined, I found that individuals exhibited lower oxidative damage than they did in warmer winters. Badgers can go into torpor in harsh weather conditions, in order to conserve energy when foraging is precluded due to frost, although this does not conserve protein (Newman *et al.* 2011). The low metabolic rate associated with torpor decreases OS levels in many species (Carey *et al.* 2003), and even increase maximum lifespan for some mammals, through reduced metabolic activity (Lyman *et al.* 1981). The down-side of decreased metabolism is the surge in oxygen consumption, and thus risk for oxidative damage, on awakening from torpor or hibernation due to increased thermogenesis (Orr *et al.* 2009).

In species that undergo short bursts of torpor rather than ongoing hibernation, there is a risk of repeated oxidative damage when re-awakening, repeatedly. Species are known to upregulate antioxidant defences to mitigate this increased oxygen consumption (Hermes-Lima and Zenteno-Savın 2002). Thermogenesis, however, also allows for enhanced antioxidant enzyme activity, which can protect against
tissue damage (Bagnyukova *et al.* 2003).

Another important factor which can increase oxidative stress is reproduction (Blount *et al.* 2015). In Chapter 4, I found that reproduction in female badgers has an oxidative cost, with oxidative damage arising from lactation. The development of a genetic pedigree assigning cubs to mothers is underway, and this will allow the attribution of actual litter sizes and specific cub identity to each female. This will ultimately reveal if male cubs incur higher OS costs to produce than females (Dugdale *et al.* 2003; *sensu* Trivers and Willard 1973). Pedigree will also enable us to investigate if reproduction has a cumulative effect on OS and OD linked to lifetime reproductive success (LRS), or if effects are resolved entirely per annual reproductive cycle. Because I only 43 females lactated over the three years of this study, I were not able to decompose reproductive interactions with OS into traits linked to 'good' or 'bad' years. In future it would be instructive to see if these data fit the general theory surrounding longevity assurance mechanisms (LAMs) where, in K-selected species, reproduction will be favoured in good weather conditions and somatic maintenance prioritised in harsh conditions (Kirkwood and Rose 1991, Campbell *et al.* In press). The trade-off between reproduction and oxidative stress has been linked in particular to the insulin/insulin-like growth factor pathway (reviewed in Tatar *et al.* 2003). It is also important to take into account carry over effects (see above and Chapter 5), where it is known from previous

studies that rainfall in the autumn affects the likelihood of reproduction in the spring. Here I propose that body condition is not the only relevant indicator of "quality", but that antioxidant defences should also be considered. I found no significant effect of ageing on OS levels in badgers (Chapter 4), despite using a longitudinal approach to expose individual changes in OS measurements over three study years. This is despite the fact that several senescent traits have been observed in badgers, such as decreased reproductive success, shortening of telomeres and reduced immune function (Buesching *et al.* 2009, Dugdale *et al.* 2011b, Beirne *et al.* 2016). I propose that in order to maximise LRS, badgers invest more in somatic maintenance as they get older, in order to reproduce again later, leaving older badgers in similar condition as younger individuals, but with lower reproductive rate (Monaghan *et al.* 2009, Campbell *et al.* In press). Due to selective mortality, weaker individuals are progressively weeded out of age cohorts, creating a sub-set of increasing fit individuals (Gaillard and Yoccoz 2003). To address this, longitudinal analyses account for that probability by investigating changes *within an individual* as it ages. Thus a poor quality individual that senesces soon after sexual maturity, or a good quality individual that senesces much later should be evident in longitudinal data. It is plausible that three years of data were insufficient to see significant declines in individual performance, compared to the relatively long maximum lifespan of badgers (15 years; Macdonald et al. 2015). Interestingly, Beirne *et al.*(2016) found significant senescence in immune capability in badgers, as well as shortening of telomeres with increased age (Beirne *et al.* 2014). Shortening of telomeres has previously been linked to increased levels of oxidative stress in several species (von Zglinicki 2002), highlighting the need for further research into how oxidative damage in ageing individuals links with telomere shortening. Figure 8.2 summarises the current

knowledge on interactions of adult OS levels with intrinsic and environmental conditions, as well as future avenues of research.

Most reviews (and indeed empirical studies) do not investigate reactive nitrogen species (RNS) as they are deemed to be less important than ROS. In this thesis, however, I investigated ability of badgers to produce nitric oxide (NO), a key RNS in innate immunity. Unlike typical mammalian macrophages (e.g. mice and cows: Adler *et al.* 1995, humans: Geller *et al.* 1993), badger macrophages do not produce NO in response to Toll-like receptor stimulation, therefore, I developed a methodology to investigate innate immune responses to pathogens using cytokine upregulation.

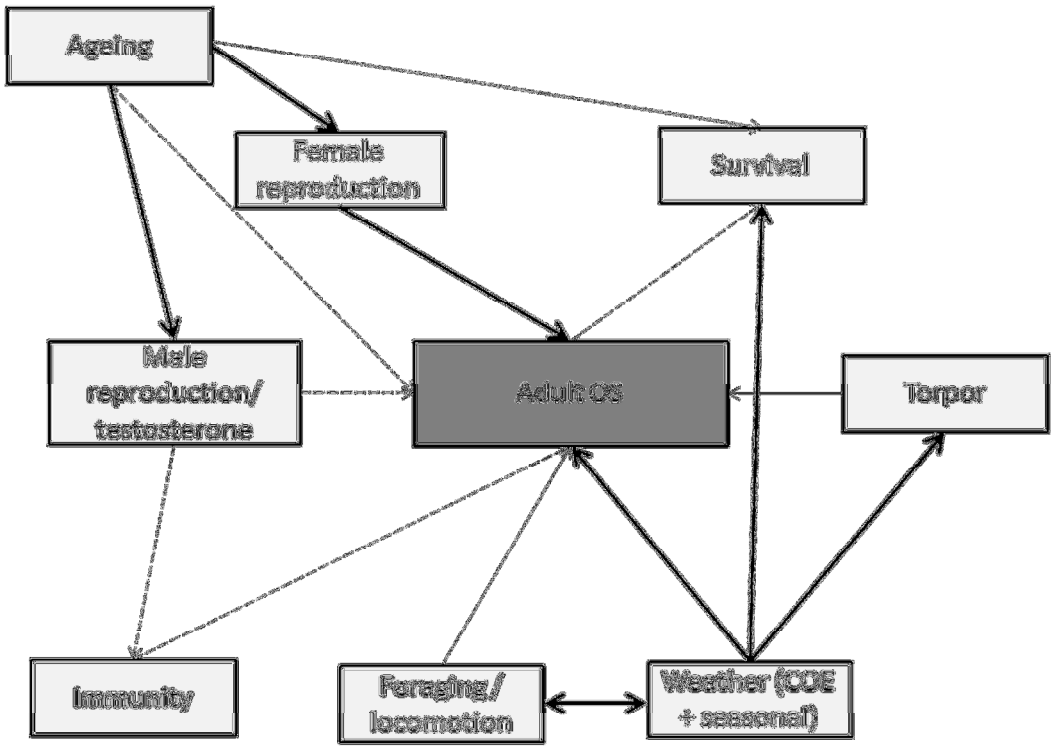

Figure 8.2 Correlates of oxidative stress in adult badgers.

Thick lines indicate results known in badgers. Thin lines indicate assumptions from known data. Dotted lines are interactions that warrant further attention in badgers, given the other interactions presented and data on other species.

Developing molecular methodologies for ecological studies is a huge field (Baker 2009, Costantini *et al.* 2010, Beaulieu and Costantini 2014) and in Chapter 6 I present the step by step development of an immunological tool box for assessing innate immune recognition of pathogens and development of an innate immune response in a wildlife species. A particular problem with tailoring molecular methods to non-standard laboratory species, is that in many cases genomes are not available (Schuster 2007). In the case of the European badger, despite its implication in maintaining bovine TB in the UK as a wildlife reservoir (Woodroffe and Donnelly In press), very few genes have been sequenced, even with regards to immunological function (but see Dalley *et al.* 2004a, Sin *et al.* 2012b). While developing species specific methods from orthologous sequences is more time consuming than using orthologous sequences themselves or non-specific assays (Ranwez *et al.* 2007), it allows for a tailored approach to a specific problem. This is of particular importance in the management of endangered species or economically important zoonosis, where quality of vaccination and management strategies are of great importance (e.g. Lesellier *et al.* 2006). The innate immune system is the first line of defence available against pathogens in vertebrates, where pathogen associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs).

As one of the main PRRs, TLRs are crucial in recognising pathogens and initiating an immune response against it. In badgers I found significant upregulation of pro-inflammatory cytokines in response to stimulation of most TLRs. TLR9, important in

the recognition of bacterial DNA, was hard to stimulate, with very high doses of *E.coli* DNA required to even get a marginal response. In addition to this apparent deficiency in response to TLR9 stimulation (which may be explained by the low levels of TLR9 mRNA in the macrophages isolated from badger blood), I found no production of nitric oxide (NO), one of the main effector molecules against intracellular pathogens, and especially required during TB infection. From this I propose that specifically tailored strategies for management of bTB in badgers should take into consideration the lack of nitric oxide production (see Chapter 7), as well as the low response to TLR9 stimulation and low levels of IL12p40 produced in response to TB stimulation. Proactive culling experiments in badgers, in an effort to control bTB in the UK led to a reduction in the number of cattle herds affected by bTB (reviewed by Woodroffe and Donnelly In press). In the areas surrounding these proactive culling zones, however, more cases of bTB were recorded in cattle herds, as badger social groups were disturbed, leading to emigration into the newly achieved low badger density areas (Godfray *et al.* 2004). As a result of these movements by potentially infected stressed and thus immune-compromised individuals, risk of cattle contagion is increased, as was previously predicted by modelling (White and Harris 1995). This epidemiological phenomenon has highlighted the need for a better management strategy than culling, as this method is expensive, highly controversial and in some cases increases the risk of bTB cases in cattle. This experiment confirms modelling results from earlier studies, which also suggest that vaccination would be a much more viable solution in the long run unless culling managed to kill off >90% of the population (White and Harris 1995). Vaccination with Bacillus-Calmette Guerin (BCG) is the only therapy approved for use in badgers, as an intramuscular injection (Lesellier *et al.* 2006). This requires capture of individuals as the oral version of the vaccine had not yet been developed for badgers

(Corner *et al.* 2010). Additionally, while BCG vaccines offer some level of protection against TB in badgers, and can potentially achieve herd immunity within badger populations (Carter *et al.* 2012b), individuals can still become infected by the pathogen, and the potential for BCG vaccination to reduce bTB prevalence on a large scale has not yet been proven (Chambers *et al.* 2011). There is thus scope for improving vaccination strategies, both from a deployment point of view as well as the specificity of the vaccine, which is an important consideration for adjuvant development and efficacy of vaccines. Adjuvants are used to increase the magnitude of a response to a vaccine, but can also be used to guide the immune system into producing the best immune response to that specific pathogen (Pashine *et al.* 2005, Coffman *et al.* 2010). In bTB vaccination, CPG (TLR9 stimulants) adjuvants have been proposed as a potential way of increasing BCG vaccination efficacy (reviewed in Buddle *et al.* 2011). Here I show that the very low response of badger macrophages to CPG motifs could strongly compromise the use of such adjuvants, and that instead adjuvants targeting TLRs for which I observed very strong pro-inflammatory responses (TLR4 / TLR5) should be used, or one that induces strong expression of IL12p40, a key cytokine in the immune control of TB (Fortin *et al.* 2007, Morahan *et al.* 2007).

The evolutionary implications of the lack of NO production, through low iNOS upregulation would also be interesting to investigate. Indeed, badgers are known to be co-infected by many pathogens at any one time (Sin *et al.* 2012a, Sin *et al.* in press), with fossoriality leading to potential for frequent re-infection (Noonan *et al.* 2015a), where sett environment and cohabitation are ideal conditions for bTB transmission (Newman & Byrne, 2016).

One hypothesis is that badgers have evolved to produce little of the potentially damaging molecule NO in order to minimise somatic damage (Nathan and Xie 1994, MacMicking *et al.* 1995). This would be an extreme form of evolutionary tolerance (Lee and Mazmanian 2010). 80% of mustelids show some level of fossoriality (Noonan *et al.* 2015a), which could have led to the evolution of this trait.

It is clear from these results that researchers cannot assume that the immune function will always work in the 'standard' way suggested by laboratory species, and that species of infectious concern will need further investigation.

In a wider ecological and conservation context, knowledge of mustelid innate immunity can inform the conservation of endangered species, at risk because of disease (see Newman & Byrne, in press) by tailoring management strategies (e.g. tailored vaccines). For example, despite being recovered from the brink of extinction, the black-footed ferret (*Mustela nigripes*) in North America is still classed as endangered by the IUCN, mainly due to diseases such as the sylvatic plague (Thorne and Williams 1988, Antolin *et al.* 2002). Similarly, Aleutian disease is also a contributing factor to the current endangered state of the European mink (*Mustela lutreola*), adding to many other vulnerabilities, such as inter-specific competition with the American mink (*Neovison vison*) and habitat restriction (Fournier-Chambrillon *et al.* 2004). With specific regard to mycobacteria, other species (non-mustelids) are also affected by tuberculosis such as the endangered Iberian Lynx (Gortázar *et al.* 2008), or brushtail possums (*Eichosurus vulpecula*) in New Zealand (Ramsey and Efford 2010), and could benefit from a similar approach to that applied to badgers, which should be investigated to see if there is an immune-deficit (iNOS) basis to their role in bTB dynamics.

Conclusion

From this thesis, I emphasise the necessity to conduct further studies examining the relationship between innate immune responses, oxidative balance and population trends under variable (wild) conditions. With more insight, conservationists will be better able to understand the role of immuno-ecology and oxidative ecology as indicators of population health. In order to maximise the benefit of these studies, a set of 'standard' OS measurements that are particularly informative for each taxa would be a major asset. In line with Beaulieu et al (2013), I therefore urge other research teams to explore ecophysiology further (i) in biological systems exhibiting different life-history traits, (ii) during different life stages, and (iii) in variable environmental conditions. In particular, I advocate a longitudinal approach "from conception until death" as the strongest approach to detect developmental changes, influences of juvenile development on adult reproductive success and survival, as well as ageing trends. For this, species with easily tractable juveniles at the time of birth, as well as known 'death dates' would be of particular use for informing the full effect of OS on life-history constraints.

Appendix A: *In situ* behavioral plasticity as compensation for weather variability: implications for future climate change

Michael J. Noonan, Chris Newman, Andrew Markham, Christina D. Buesching, Kirstin Bilham, David W. Macdonald

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A.1 **Abstract**

Climate change places species in evolutionarily novel conditions that could result in serious population declines, or extinctions, over the coming 50-100 years. Despite climatic effects on species biogeographic distributions being well documented, far less research has considered individuals' capacity to accommodate altered conditions *in situ*. Here, we utilize high-resolution tracking of the European badger, as a model species, to examine weather-driven modification of activity and behavior through the critical autumn season. This study was conducted in Wytham Woods, UK (GPS reference: 51°46′26″N; 1°19′19″W); a 424-ha mixed semi-natural woodland designated a Site of Special Scientific Interest (SSSI). Badgers live in underground burrows, termed setts, which provide thermal refugia; thus exposure to unfavorable conditions can be adjusted to optimize energy budgets. Activity was assessed using new accelerometer and active-RFID transponder technology. We first applied Bayesian mixed models to investigate the relationship between weather variables and: i) activity duration; ii) Overall Dynamic Body Acceleration (ODBA); and iii) ranging behavior. We then used behavioral response models to parameterise simulations of how future climate change scenarios might affect behavior. Bayesian inference showed that inter-individual differences in activity were promoted significantly by temperature, but inhibited by day length, relative humidity, and rainfall. Furthermore, warm, humid conditions led badgers to make significantly more visits to neighbouring group dens, especially individuals in better body-condition. Modeled against IPCC climate change scenarios, the milder and drier conditions projected for the next century would likely cause badgers to maintain elevated activity levels into winter months. According to our projections, this would necessitate an increase in energetic expenditure by as much as 31% per day.

Less mobile species must compensate for climate change *in situ* via behavioral plasticity, rather than relocating to maintain continuity with their current bioclimatic niche. Badgers proved versatile, although projected behavioral responses did not follow a linear trajectory. Future climate change will likely drive substantial additional autumnal activity, and ultimately winter food supply limitations will cease to satisfy increased energy requirements, defining tolerance threshold.

Keywords: HIREC, Human-Induced Rapid Environmental Change, Weather variables, Climate projection, Behavioral compensation, Energetics, Adaptation

A.2 **Introduction**

Rapid, anthropogenic climate change (IPCC 2014) risks exposing species to evolutionarily novel situations they may be unable to accommodate (Palumbi 2001). This may ultimately force range shifts (Walther et al. 2002), or cause serious population declines, and even extinctions (Stork 2010). Although even quite rapid rates of natural climate change are not unprecedented in the paleontological record (Dansgaard et al. 1993), species' capacities to accommodate current climate change are also impacted by other Human-Induced Rapid Environmental Change (HIREC) pressures (Sih et al. 2011), such as habitat loss, fragmentation, and/or loss of ecosystem biodiversity (Venter et al. 2006, Sih et al. 2011, Bellard et al. 2012). Failure to compensate climatic change adequately will result in impaired energetic balance (Chevin et al. 2010), and/or reduced fitness (Hellmann and Pineda-Krch 2007), potentially impacting the species' realised niche (Quintero and Wiens 2013). Furthermore, responses are typically non-linear, where exceeding 'tipping points' can trigger disproportionately severe consequences (Doak and Morris 2010). Despite well documented coarse-scale

adaptations to global climate change (Walther et al. 2002), a finer-scale understanding of more nuanced individual behavioral responses is lacking (discussed by Newman and Macdonald 2015). It is therefore crucial to understand species immediate behavioral responses to changing conditions from a mechanistic perspective (Sih et al. 2011).

To maintain bioclimatic niche continuity, and to allow existing behaviors to continue to function, more mobile species tend to shift their use of habitat mosaics, or relocate entirely (reviewed in Walther et al. 2002, Barnosky and Kraatz 2007); a trend also prevalent in the fossil record (Fernández and Peláez Campomanes 2005, Lister and Stuart 2008). In contrast, less mobile species, and/or those tied to specific habitats (e.g., semi-aquatic, -fossorial, and -scansorial specialists, or central-place foragers), have to contend with changing local conditions *in situ* (e.g., Campbell et al. 2012). These species must accommodate change either through differential genetic trait selection (Visser 2008), changes in population dynamics (Walther et al. 2002, Macdonald et al. 2010), or through behavioral plasticity (Kearney et al. 2009, Noonan et al. 2014).

Genotypic adaptation, via Darwinian selection, takes generations to occur (Barnosky and Kraatz 2007). Species already near their physiological limits (Hoffmann and Sgrò 2011), or with longer inter-generational times (Rosenheim and Tabashnik 1991), are therefore especially vulnerable to environmental change. Consequently, behavioral plasticity provides the most immediate tactical response to changed conditions (Bradshaw and Hardwick 1989, Walther et al. 2002, Kearney et al. 2009, Sih et al. 2010, 2011). Behavioral responses are also less permanent than genetic selection, and thus better suited to temporary, or stochastic changes (Bradshaw and Hardwick 1989). Not all behaviors can be modified, however, particularly those tied tightly to ensuring reproduction/ fitness (Visser 2008), or dependent on inter-specific relationships (Cahill et al. 2012). In this respect, generalist species are more likely to

respond through individual plasticity within their lifetimes, while rigid strategists are biased toward differential selection for phenotypes better fitting transformed conditions (Visser 2008).

A.2.1 **Model species: The European badger**

European badgers (*Meles meles*, henceforth 'badgers') have provided an informative model for studying how population dynamics (Macdonald and Newman 2002, Macdonald et al. 2010, Nouvellet et al. 2013, Byrne et al. 2015) and behavior (Noonan et al. 2014, 2015b) respond to weather variation and climate change. They have a pan-European distribution, (Johnson et al. 2002), with ecologically similar congenerics occurring through northern Asia (*M. leucurus; M. canescens*) into Japan (*M. anakuma*). In lowland Britain, badgers live in groups of 5 to 25 individuals (Macdonald et al. 2015). They are adaptable generalists (Newman et al. 2011), tied to fossorial dens (termed 'setts') within forest and forest/agrarian mosaic habitats (Macdonald et al. 2015), and generally not prone to extensive dispersal (Macdonald et al. 2008, but see Byrne et al. 2014). Much of their climatic sensitivity is a result of their feeding ecology, where earthworms (principally *Lumbricus* spp.) are a major component of their diet (Goszczyński et al. 2000, Macdonald et al. 2015). Earthworms are sensitive to micro-climatic conditions (Curry 2004, Nuutinen and Butt 2009), and their distribution and availability to consumers is highly dependent on prevailing weather conditions (Macdonald 1983, Kowalczyk et al. 2003). Furthermore, badgers can decide to remain underground in their thermally-stable setts (Kaneko et al. 2010) during harsh weather conditions (Noonan et al. 2014, 2015a) and enter torpor if poor weather and low food availability persist (Newman et al. 2011). In addition to foraging, there are other individual-specific drivers motivating badger activity, such as territorial (Tinnesand et

al. 2015), social (Buesching et al. 2003), and mating (Annavi et al. 2014) interactions. Therefore, each individual must trade-off these behaviors against net losses to body-condition incurred by activity on thermally challenging nights (Kowalczyk et al. 2004, Noonan et al. 2014, 2015a).

Badger survival dynamics are particularly sensitive to autumnal conditions (Macdonald et al. 2010, Nouvellet et al. 2013) linked to their need to accumulate fat reserves (Noonan et al. 2014), prior to potential winter frost, snow and low(er) food supply (Kowalczyk et al. 2003). Failure to gain sufficient body-condition during the autumn reduces their potential to use torpor to see them through winter periods, when activity carries a high energetic cost (Fowler and Racey 1988, Newman et al. 2011). On this account, we conducted this study across the autumnal months.

To investigate fine-scale effects of weather conditions on individual activity patterns, we equipped badgers with tri-axial accelerometers (Wilson et al. 2008), able to derive overall dynamic body acceleration (ODBA; Wilson et al. 2006) as a measure of energy expenditure. We also used an animal-borne active-RFID tracking system, combined with a sensor network across the landscape, to log the locations of multiple badgers in near real-time (Noonan et al. 2015a). In addition, we inserted thermal probes into setts, to measure their potential as thermal refugia (Kaneko et al. 2010).

We posit that, to maintain energetic equilibrium, badgers will adjust their above-ground activity versus remaining within the refuge of their setts, and alter their roaming behavior, in response to prevailing conditions. Because badgers cannot survive within their setts indefinitely without leaving to forage, we anticipate that individuals with lower fat reserves will be more motivated to feed under sub-optimal weather conditions than fatter badgers (*sensu* Noonan et al. 2014). We also consider how behavioral responses are modified by sex, and reproductive status.

A.2.2 **Climate change predictions**

We proceed to model what effects future projected climate change scenarios (Murphy et al. 2009, IPCC 2014) might have on badger activity regimes. We consider whether these behavioral changes are sustainable with regard to food supply thresholds, or if modified activity regimes risk exposing badgers to other mortality factors, such as road traffic accidents (Macdonald et al. 2010). From these findings, we extrapolate to similar vulnerabilities other low-mobility species may encounter in response to climate change.

A.3 **Methods**

This study was conducted in Wytham Woods, Oxfordshire (GPS reference: 51°46′26″N; 1°19′19″W); a 424-ha mixed semi-natural woodland designated as a Site of Special Scientific Interest (SSSI), and managed by the University of Oxford. The resident, high-density badger population comprised 23 social groups with ca.145 adults and 52 cubs in 2014 (Noonan et al. 2015b).

As part of a long-term research program, a subset of 15 badgers (6 males and 9 females; Table 1), resident at three adjacent social groups, were caught, sedated, sampled, and measured (for details see Macdonald et al. 2015). From these morphometrics, we calculated the ratio of body weight to length (ln mass:ln length), to generate Body-Condition Indices (BCI) as a measure of stored body-fat (Noonan et al. 2014). Following Woodroffe and Macdonald (1995, see also Tinnesand et al. 2015), female reproductive status was inferred from vulva condition; classified as oestrus (vulva swollen and moist), or non-oestrus (vulva flat and dry). All males had scrotal testes when collared in August and inferred to be in breeding condition (Buesching et

al. 2009). In autumn, however, both sexes enter a period of reproductive quiescence (Woodroffe and Macdonald 1995, Buesching et al. 2009), thus reproductive motivation likely changed over the study period. Nevertheless, there are distinct physiological costs associated with maintaining reproductive condition (Woodroffe and Macdonald 1995, Buesching et al. 2009) that persist after the mating season itself, incurring ongoing costs throughout this study.

Captured badgers were fitted with custom-built tracking tags [39 mm (l) x 22 mm (w) x 12 mm (h)], including tri-axial accelerometers and active radio-frequency identification (aRFID), powered by CR123A lithium batteries (see Noonan et al. 2015a). Tags were hermetically sealed in epoxy resin, and mounted onto commercially available padded leather dog collars, with a complete assembly weight of 95 g. Henceforth, individual badgers are referred to by their unique collar ID. After handling and collaring, badgers were held in a recovery room for 3 h, before being released at the locations where they were captured (Macdonald et al. 2015).

A.3.1 **Quantifying energy expenditure**

Accelerometers sampled relative acceleration continuously, at a frequency of 8 Hz, across each of three axes (heave, surge, and sway). Data were compressed and stored onboard collars, and communicated wirelessly to base-stations (see below), making it unnecessary to recapture individuals for data collection. From these accelerometer data, we calculated two inter-dependent variables, which addressed distinct behavioral decisions: i) ODBA (in *g*), which provides an accurate proxy for energy expenditure (see Wilson et al. 2006); and ii) 'activity', which gives a binary measure of the badgers' pattern of above-ground activity versus inactivity below ground. Activity was calcuated based on an ODBA threshold value of 0.313 *g* (Noonan et al. 2014). Above this

threshold, readings were consistent with activity; below it individuals were inactive (for full details see Appendix S1 in Supporting Information). Data were collected from August 16, 2014, until Nov 7, 2014, by which point all batteries had run out. Across all 10 animals, over $3.5x10^8$ accelerometer data points were generated over 522 badger days.

A.3.2 **Quantifying movement patterns**

Due to the limitations of utilising Lagrangian tracking technologies (i.e., following individuals continuously) to track multiple individuals simultaneously in dense vegetation and when underground (Noonan et al. 2015a), we used a Eulerian sampling approach (i.e., logging visits to specific receivers in the environment, positioned at all setts used by this sub-population).

The three neighbouring focal social groups occupied ca. 49 ha and included nine setts (three main, and six outlying; Fig. 1). Each group had a fairly stable membership and distinct range, delineated by a series of inter-group latrines (Tinnesand et al. 2015) From trapping histories, all groups within the Wytham Woods population exhibit high inter-conectivity (Macdonald et al., 2008), and badgers frequently trespass temporarily into neighbouring group ranges (Macdonald et al. 2008, 2015); also evidenced by genetic analyses, revealing 48% extra-group paternity (Annavi et al. 2014). These specific focal groups were thus selected because each had direct access to earthworm-rich pasture, decreasing the likelihood that inter-group visits were related to foraging and instead implying a social function.

The 2.4 GHz 802.15.14 (Zigbee) base-station receivers we installed at each sett were powered by a 12 V battery (Noonan et al. 2015a). Base-stations received aRFID tag signals, broadcast every 3 s, whenever an animal was within a 20-25 m radius

detection range. When a collar was detected, an entry (consisting of collar ID, time-stamp, and received signal strength) was generated. Buffered data were transferred from collars to base-stations, with two-way handshaking (i.e., a network transmission control protocol where data packets are exchanged reciprocally between devices) and saved for later retrieval. Due to the way setts were distributed within the study area, it was possible for an individual to be within the detection range of multiple base-stations simultaneously. In these instances, a badger was recorded at the location where it registered the strongest signal. In total, 103,797 (re-)visits to setts were recorded.

Given the importance of photoperiod on badger activity (Noonan et al. 2014), we apply the term '*per diem*' to designate a full circadian cycle, avoiding confusion with the dual meaning of the term 'per day'. Because there was substantial variation in the number of sett sites individuals visited each night, and their frequency of (re-)visits, we used sett proximity data to calculate *per diem* utilisation distributions (henceforth 'inter-sett visits'), using the kernel Brownian bridge method in the *R* package "*AdehabitatHR*" (Calenge 2011). For each badger, the smoothing parameter σ^1 was calculated using the maximum likelihood approach developed by Horne et al. (2007), while σ^2, a measure of the imprecision of relocations, was set to 50 m (i.e., the maximum aRFID detection diameter). Although limited by the sub-sampling inherent in the Eulerian tracking approach, these utilisation distributions provide a robust minimum estimate of inter-sett visit rate.

A.3.3 **Autumnal weather conditions**

Meteorological records were obtained from the UK Environmental Change Network (ECN) weather station in Wytham Woods (terrestrial site T08). To quantify the effects of contemporaneous weather on badger activity, we decomposed these weather data

into four variables, known to influence badger behavior (Johnson et al. 2002, Macdonald et al. 2010, Noonan et al. 2014, 2015b): (1) hourly solar radiation, providing a measure of photoperiod (in W/m^2), with the additional subtle benefit of including cloud cover influences on dusk emergence; (2) mean hourly relative humidity (in %); (3) mean hourly air temperature (in °C); and (4) total hourly rainfall (in mm).

In addition, in Appendix S2 we present analyses of trends in historic autumnal weather data from 1853 to 2015, providing the range of conditions this population's ancestors were exposed to, shaping their selection. In Appendix S3, we present analyses of the sett micro-climate over the course of the study period.

A.3.4 **Descriptive analyses**

All statistical analyses were conducted in the *R* environment (version 3.0.3; R Core Team 2014). We undertook an initial descriptive analysis, using analyses of variance (ANOVA) and covariance (ANCOVA), as well as Pearson's correlations ($\alpha = 0.05$). To summarise the variance contingent on weather, activity, and energy expenditure variables, as well as individual traits, we ran a principal component analysis (PCA), performed over mean *per diem* data (n = 362). Here, sex was included as a supplementary qualitative factor, without rotation; instances with missing values were excluded.

A.3.5 **Bayesian inference**

To investigate the relationship between behavioral response variables and weather variables, we applied a Bayesian mixed models approach across mean hourly data (n = 12,546), using the *R* package '*MCMCglmm*' (Hadfield 2010). Due to the uneven spatial

distribution of monitored setts, behavioral decisions had the potential to be biased by geography and relative proximity of neighbouring base-stations. Similarly, sett visits involving longer distances require more energy. To address this, we included the random effect of group membership in all of our models. To test whether activity and energy expenditure responded to variation in weather or individual BCI, sex, and reproductive condition, two predictive models were fitted, with 'individual' and 'group' included as random effects:

i) activity = *f* (solar radiation + temperature + humidity + rainfall + sex + reproductive status + BCI)

ii) ODBA = *f* (solar radiation + temperature + humidity + rainfall + sex + reproductive status + BCI)

To examine movement patterns and inter- sett visits, the following model was fitted, with individual, and group included as random effects:

iii) inter-sett visits = *f* (solar radiation + temperature + humidity + rainfall + sex + reproductive status + BCI)

Following Hadfield (2010), we used uninformative, inverse-gamma prior distributions. We used a binomial distribution to model activity data, whereas ODBA, and inter-sett visits were modeled against a Gaussian distribution with an identity link function. All parameters were mean centred prior to analyses. To correct for a positive skew, inter-sett visit data were log-transformed. The number of iterations, thinning interval and the burn-in period were determined using diagnostic tests in the *R* package '*coda*' (Plummer et al. 2006). Convergence between model chains was verified using the Geweke diagnostic (Geweke 1992).

A.3.6 **Climate change projections**

We used activity data to parameterise simulations of how future climate change might affect badgers. Based on the IPCCs SRES emissions scenarios (Nakicenovic and Swart, 2000), we obtained UK Climate Projections 2009 (Murphy et al. 2009) for the 25 km^2 area around Wytham Woods. These data exist as mean monthly values for seven overlapping, 2-decade time-periods (from 2010 to 2099), over a wide probability range. We simulated responses to both, a low emissions (i.e., the B1 scenario, which predicts 500 to 600 ppm CO_2, a 1.1 to 2.9 °C rise in mean temperature, and no significant trends in precipitation) and a high emissions scenario (i.e., the A1F1 scenario, which predicts 550 to 750 ppm CO_2, a 2.4 to 6.4 °C rise in mean temperature, a 0 to 5% decrease in relative humidity, and no significant trends in precipitation).

We then applied the '*predict*' function in the *R* environment (R Core Team 2014) to our three models to predict how these activity variables would respond to projected weather conditions per period, detailed in Appendix S4. We note, however, that although response trends can be considered as robust, both our parameter estimates and climate predictions are subject to modelling error, and therefore interpretations should be made cautiously.

A.4 **Results**

A.4.1 **Summary of activity and movement data**

Badgers were active on average 9.7 ± 2.1 SD hours *per diem*, and, superficially (but see below) there was no evidence of a trend in mean activity over the study period ($r = -0.01$, $n = 86$, $P = 0.94$). There were significant differences in duration of *per diem* activity between individuals ($F_{[9,512]} = 9.16$, $P < 0.005$; Fig. 2a), but no difference between sexes ($F_{[1,520]} = 0.01$, $P = 0.92$).

Individuals exhibited significant differences in *per diem* ODBA expenditure ($F_{[9,512]} = 41.91$, $P < 0.005$), with a significant sex effect ($F_{[1,520]} = 22.8$, $P < 0.005$), where males had a lower *per diem* ODBA than females. As autumn progressed, badgers visited significantly fewer setts ($r = -0.24$, $n = 78$, $P = 0.03$). Inter-sett visit rates differed significantly between individuals ($F_{[9,648]} = 17.09$, $P < 0.005$; Fig. 2b), with males visiting significantly more setts than did females ($F_{[1,715]} = 25.33$, $P < 0.005$).

Only the first four components warranted retention in a PCA across mean *per diem* data, which explained a cumulative 68.7% of the variance (Table 2). From component factor loadings, reproductive status, activity/ODBA budgets, and weather variables were the most influential parameters. Projecting these data into the reduced dimension space defined by PC 1 (termed the "energy budget component") and PC 2 (termed the "weather component") revealed minimal overlap between sexes, within which individuals in different reproductive condition grouped into relatively distinct clusters (Fig. 3).

A.4.2 **Modeling movement patterns**

From Bayesian inference (Table 3), weather conditions were crucial for predicting the amount of time spent active, versus time spent in thermally stable setts (see Appendix S3). Posterior parameter estimates revealed that duration of activity was promoted significantly by temperature and inhibited significantly by solar radiation, relative humidity, and rainfall. In contrast, when modeling ODBA, although the significant negative relationship with solar radiation, relative humidity and rainfall persisted, the positive effect of temperature became non-significant. From posterior parameter estimates, temperature, humidity and BCI had significant positive effects on the rate of

per diem inter-sett visits. Individuals with higher BCI tended to visit more setts, where warm, humid conditions promoted inter-sett visits.

A.4.3 **Climate change projections**

The changes predicted by climate scenarios through the 21st Century appear likely to drive a substantial change in badgers' autumnal behavioral phenology (detailed in Appendix S4). Under the low emissions scenario, milder and less humid conditions could cause a 4% ± 22.7 SD mean increase in activity duration to be maintained into the winter months (Fig. 4a). This would necessitate summer levels of ODBA being maintained into winter, requiring 6% ± 8.9 SD more ODBA '*per diem*' than present day (Fig. 4b). Under this scenario, activity and ODBA adjustments were accompanied by only a mean 1% ± 7.2 SD increase in inter-sett visits (Fig. 4c). In contrast, under a high emissions scenario, only a 2% ± 23.8 SD mean increase in activity duration was predicted (Fig. 4d), but, counter-intuitively, accompanied by a 7% ± 8.9 SD increase in ODBA expenditure (Fig. 4e). Interestingly, this scenario predicted a 3% ± 9.0 decrease in inter-sett visits (Fig. 4f).

A.5 **Discussion**

Ultimately, how populations cope with climate change is a function of how individuals respond to cumulative, contemporary weather stresses (Visser 2008, Noonan et al. 2014, see also Blumstein and Berger-Tal 2015). Schloss et al. (2012) predict that, for the western hemisphere, an average of 9.2% of mammals at any given location will be unable to respond to climate change adequately – in some regions up to 39% may be unable to keep pace. Similarly, the biogeographic ranges of 87% of mammalian species

are expected to undergo reductions, where for many this will be due to limited dispersal abilities, as opposed to reductions in area with suitable climate (Newman and Macdonald 2015). Within these predictions, however, lies a core assumption; that species will go on trying to operate in the same way as at present, despite changes to their bioclimatic niche. This assumption is clearly invalid – tactically, all species will, to some degree, attempt to exploit their innate behavioral plasticity to accommodate the cascade of stressors resulting from climate change (Bradshaw and Hardwick 1989, Chevin et al. 2010, Noonan et al. 2014). Studies quantifying how behavioral plasticity underlies mechanistic responses to climatic change are, however, few (Blumstein and Berger-Tal 2015). Ultimately, this leads to a lack of understanding how weather conditions affect behavior and activity at the individual level for many species (reviewed in Newman and Macdonald 2015).

To begin to redress this information gap, we report how the European badger, a K-selected generalist with a wide biogeographic range, uses behavioral plasticity to accommodate variation in weather. Even given the small geographic extent occupied by this sub-population, and the relative homogeneity of their forest habitat, we observed significant response heterogeneities between individuals not explained by BCI, sex, reproductive status, or group membership. Badgers exhibited three primary responses: first, when conditions were mild and humid, but without heavy rainfall, they were active for less time. This implies they could satisfy their dietary needs quickly and effectively (e.g., Kowalczyk et al. 2003) when conditions were conducive to earthworm emergence (Macdonald 1983, Curry 2004). Second, when it was drier and mild, badgers were active for extended periods – likely because more foraging effort was required when earthworms were less abundant, but without activity incurring substantial thermoregulatory costs. Finally, under colder conditions, badgers were significantly less

active – extended periods of activity risk net-negative energetic returns during conditions of low(er) earthworms availability, particularly under harsh conditions (Noonan et al. 2014). These tactical responses suggest badgers attempt to optimise their activity in response to prey availability and the risks of thermoregulatory losses above-ground.

Nevertheless, irrespective of prevailing weather and the need to forage, species must weigh decisions on commitments to other activities. For example, we found up to four hours difference in individual activity per night often occurred between dyads. Furthermore, on some nights, certain badgers did not emerge from their thermally stable burrows (see Appendix S3) at all, while others were active for a normal (ca. 8 hrs) duration. These differences may reflect differences in energy-based motivation, and/or differences in behavioral objectives. In this regard, we also found a positive relationship between temperature, humidity and the rate of inter-sett visits. This suggests that when badgers can readily meet their energetic requirements can be met without thermoregulatory costs, more energy can be invested in social activities. Furthermore, although we found no significant relationship between BCI and activity; those badgers with higher BCI, of both sexes, tended to make more inter-sett visits; where extra-group mating is known to enhance breeding success (Annavi et al. 2014).

The tactical reliance on innate behavioral plasticity evidenced in this study reveals, mechanistically, that badger responses to prevailing weather have, thus far, been sufficient to ensure the perpetuation of this population (see also Macdonald et al. 2010, Nouvellet et al. 2013). Badgers have a recorded history of occupying this study-site for centuries (Macdonald et al. 2015), likely since the Holocene interglacial began (Sommer and Benecke 2004). Although our current study took place in a comparatively low weather-stress year, meteorological records for this site commencing in 1853 reveal

a number of extreme weather event years (i.e., mean values of weather parameters falling more than two standard deviations from long-term means; see Appendix S1). These harsh years likely impacted the population severely (Nouvellet et al. 2013), but illustrate the resilience with which this species can survive weather stress at the population level. In terms of trends, there has been an incremental, 1.7 °C increase in temperature (consistent with global trends; IPCC 2014), which, to date, has generally benefited population success (Macdonald and Newman 2002; Newman and Macdonald 2015).

Few projections of future species-success take behavioral plasticity into account (e.g., Keith et al. 2008). We demonstrate that warming conditions in the future may promote extended periods of autumnal badger activity. Crucially, however, these modeled activity responses exemplify why linear response trajectories should not be assumed (Doak and Morris 2010). We project the IPCC's low emissions scenario will result in increased activity, ODBA, and inter-sett visits, whereas the high emissions scenario involves a relative decrease in activity and inter-sett visits, but an increase in ODBA. This ODBA increase would amount to 31% more mechanical energy expenditure *per diem* than current levels. In this respect, Turbill et al. (2011) highlight that hibernation is generally associated with higher annual survival, longer life spans, slower reproductive rates, later maturity, and longer generation times when compared with similar sized non-hibernating species. This strategy is, however, is generally compromised when winters become too warm for metabolic rates to remain sufficiently suppressed (Newman and Macdonald 2015). For instance, Pretzlaff and Dassmann (2012) found that dormice (*Muscardinus avellanarius*) do not compensate for changes in ambient temperature, and are thus more likely to deplete fat reserves before the end of hibernation with warmer temperatures. Furthermore, arousal also occurred more

frequently during warmer winters, resulting in significantly increased energy expenditure. Similarly, climate change effects can cause bats to awaken from hibernation during periods of low food availability, depleting their energy reserves and leading to mortality (Foley et al. 2011). For many mammals therefore, changes in phenology, via the mechanisms evidenced in this study, could cause specific calorific requirements to be out of synchrony and/or balance (Walther et al. 2002) with seasonal food availability. If food supply cannot keep pace with this heightened rate of energy expenditure through the winter months, this will ultimately compromise species' population dynamics and reduce population density (Walther et al. 2002). It should be noted however, that there are other complicating factors that are not captured in these models; hibernation/torpor are not only a response to weather conditions. These processes are also under endocrinological control via day-length, operating through the pineal hormone melatonin (Bechtold et al. 2012). Ability to respond to changing autumnal weather would thus have an interactive hormonal component, where day-length variation stays fixed with latitude.

There are also confounding corollaries of greater winter activity. Macdonald et al. (2010) describe how modification of activity patterns and ranging behaviour with milder winter temperatures are linked to higher rates of badger road traffic accidents (RTAs) in the urban UK, which are the primary cause of adult mortality. Similarly, an analysis by Glista et al. (2008) of more than 60 vertebrate species revealed that temperature was the primary predictor of RTA mortality. Interestingly however, Rolandsen et al. (2011) found that for moose (*Alces alces*) populations, warmer winters result in fewer RTAs. They speculated that moose are cold-adapted, and thus maintain higher levels of activity at lower temperatures, demonstrating the importance of

accounting for species- and situation-specific behavioural plasticity, precluding a '*one size does not fit all*' framework.

A meta-analysis by Hendry et al. (2008), examining over 3000 instances of phenotypic change rates, concluded that behavioral plasticity proved the most important response to climate change. Understanding the ability to compensate climate change *in situ*, through plastiticity in behavioral tactics (Hendry et al. 2008), must become a climate-ecology and conservation priority (Sih et al. 2010, Wong and Candolin 2015, Newman and Macdonald 2015, Blumstein and Berger-Tal 2015). As we have shown, this is especially important in regions where proximate weather conditions are highly variable, such as the UK (UKCP09; Murphy et al. 2009), where the flexibility afforded by behavioral responses are most effective (Nouvellet et al. 2013).

A.6 **Acknowledgments**

Without the generous support of The Peoples Trust for Endangered Species this core study would not have been possible. We would also like to thank Biotrack Ltd. for their expertise in manufacturing and assembling our custom tracking collars. MJN was supported by the Rhodes Trust and an NSERC postgraduate scholarship, and AM was supported by EPSRC Undertracker EP/I026959/1. All procedures undertaken for this study were covered by both Natural England (2014-5710-SCI-SCI), and Home Office licenses (PPL 30/2385), and approved by the University of Oxford's Animal Welfare and Ethical Review Board, and all personel handling animals were qualified Personal Individual Licence (PIL) holders.

Table 1 Summary information of collared animals. BCI represents ln scaled mass:length; reproductive status was determined by testes decent in males, and vulva condition in females.

Deployment Date	Collar Number	Sex	BCI	Reproductive Status	Operational Days
12/08/14	1	M	0.326	Descended	50
14/08/14	2	F	0.333	Non-oestrus	23
14/08/14	3	M	0.297	Descended	64
15/08/14	4	M	0.331	Very descended	-
15/08/14	5	M	0.331	Descended	65
15/08/14	6	M	0.342	Very descended	54
15/08/14	7	F	0.304	Non-oestrus	84
16/08/14	8	F	0.271	Non-oestrus	33
16/08/14	9	F	0.333	Oestrous	-
16/08/14	10	F	0.310	Non-oestrus	-
16/08/14	11	F	0.325	Non-oestrus	-
19/08/14	12	M	0.303	Very descended	54
19/08/14	13	F	0.331	Non-oestrus	47
19/08/14	14	F	0.322	Oestrous	48
19/08/14	15	F	0.288	Oestrous	-

Table 2 Summary of the first four components of a principal component analysis performed over mean *per diem* data, with sex included as a supplementary qualitative factor. ODBA represents accelerometer derived overall dynamic body acceleration (*g*), an accurate proxy for energy expenditure; and BCI the ratio of body weight to length (ln mass:ln length).

	PC 1	**PC 2**	**PC 3**	**PC 4**
Eigenvalue	2.49	2.14	1.44	0.91
% of variance explained	24.94	21.42	14.42	9.07
Cumulative % of variance explained	24.46	45.36	60.78	69.84
Factor loadings				
Individual	-0.17	-0.25	-0.17	0.77
BCI	0.05	< 0.01	0.65	-0.07
Reproductive status	-0.34	-0.31	0.20	0.20
Hours active	0.29	0.46	< 0.01	0.41
ODBA	0.41	0.48	0.02	0.11
Temperature	0.35	-0.20	0.15	0.37
Relative humidity	-0.43	0.33	0.06	< 0.01
Rainfall	-0.36	0.35	0.02	0.06
Solar radiation	0.39	-0.36	-0.07	-0.18
Inter-sett visits	0.02	-0.02	0.69	0.09

Table 3 Relationship between weather metrics, individual attributes; sex, Body Condition Indices (BCI), and reproductive status, and the response variables i) activity; ii) ODBA; and iii) *per diem* inter-sett visits in the linear model. Posterior coefficient estimates (β), and their 95% confidence intervals (CI) are presented. Asterisks denote variables that differed statistically from zero (based on 95% confidence intervals). ODBA represents accelerometer derived overall dynamic body acceleration (g); and BCI the ratio of body weight to length (ln mass:ln length). Note: the activity model had 6.0×10^7 iterations, with a 2.4×10^3 burn-in period and a thinning interval of 3.3×10^2; the ODBA model had 6.0×10^7 iterations, with a 2.0×10^3 burn-in period and a thinning interval of 1.1×10^2; and the inter-sett visit model had 4.0×10^7 iterations, with a 2.0×10^3 burn-in period and a thinning interval of 1.0×10^2.

	Activity			**ODBA**			**Inter-sett visits**		
	B	**lower CI**	**upper CI**	***B***	**lower CI**	**upper CI**	***β***	**lower CI**	**upper CI**
Fixed terms									
Intercept	-3.90**	-5.76	-2.00	0.01	-1.60	1.65	-0.03	-0.09	0.03
Solar radiation	-0.04***	-0.05	-0.04	-0.06***	-0.08	> -0.01	> -0.01	> -0.01	< 0.01
Temperature	0.09***	0.07	0.11	< 0.01	> -0.01	< 0.01	0.01**	< 0.01	0.02
Humidity	-0.05***	-0.06	-0.04	> -0.01***	> -0.01	> -0.01	< 0.01*	< 0.01	< 0.01
Rainfall	-0.29*	-0.54	-0.04	-0.06***	-0.08	-0.04	-0.01	-0.01	< 0.01
BCI	4.73	-34.64	45.06	-0.06	-34.48	32.87	3.12**	1.48	4.63
Reproductive status	-0.73	-1.96	0.48	-0.08	-1.10	0.99	0.02	-0.03	0.08
Sex	0.08	-1.43	1.54	-0.01	-1.29	1.23	0.05	-0.03	0.12
Random terms									
Individual	0.48**	0.06	1.25	0.34**	0.05	0.87	< 0.01*	< 0.01	< 0.01
Group	2.04*	0.06	5.89	1.51*	0.06	4.56	< 0.01*	< 0.01	< 0.01
Residual variance	0.15***	0.06	0.28	0.05***	0.05	0.05	0.03***	0.02	0.03

*** $p < 0.001$,** $p < 0.01$,* $p < 0.05$

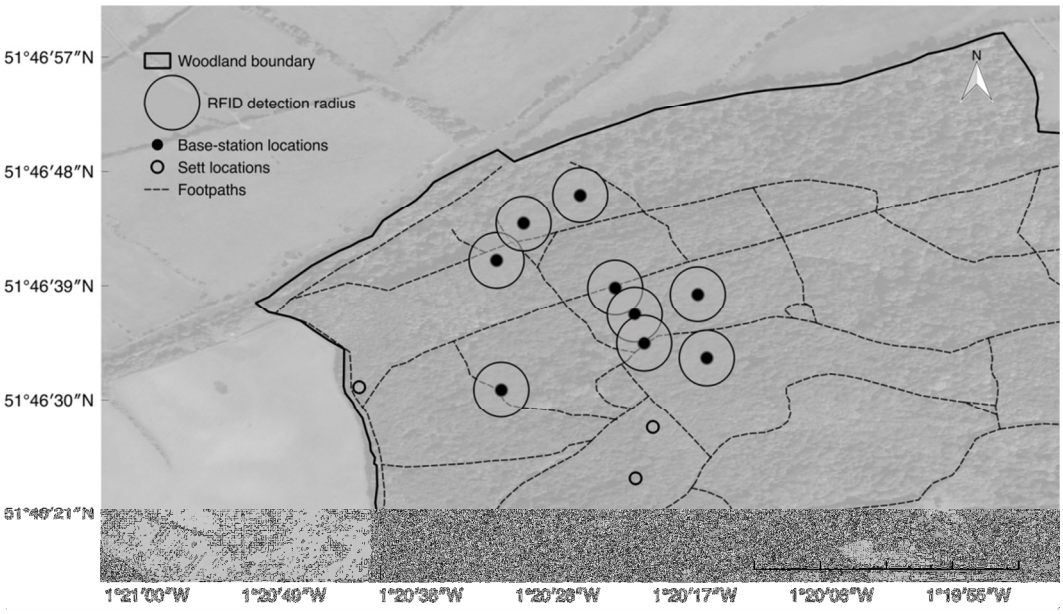


Figure 1 Map of the study area within Wytham Woods, UK (GPS reference: 51°46′26″N; 1°19′19″W), depicting the locations of aRFID base-stations at monitored sett sites, as well as unmonitored, nearby sett sites.

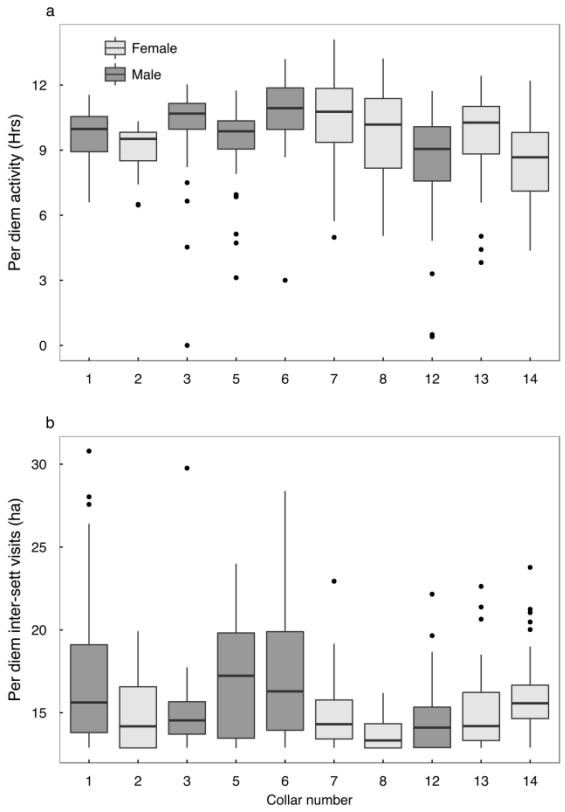


Figure 2 Boxplots of *per diem* a) activity; and b) inter-sett visits of each collared badger.

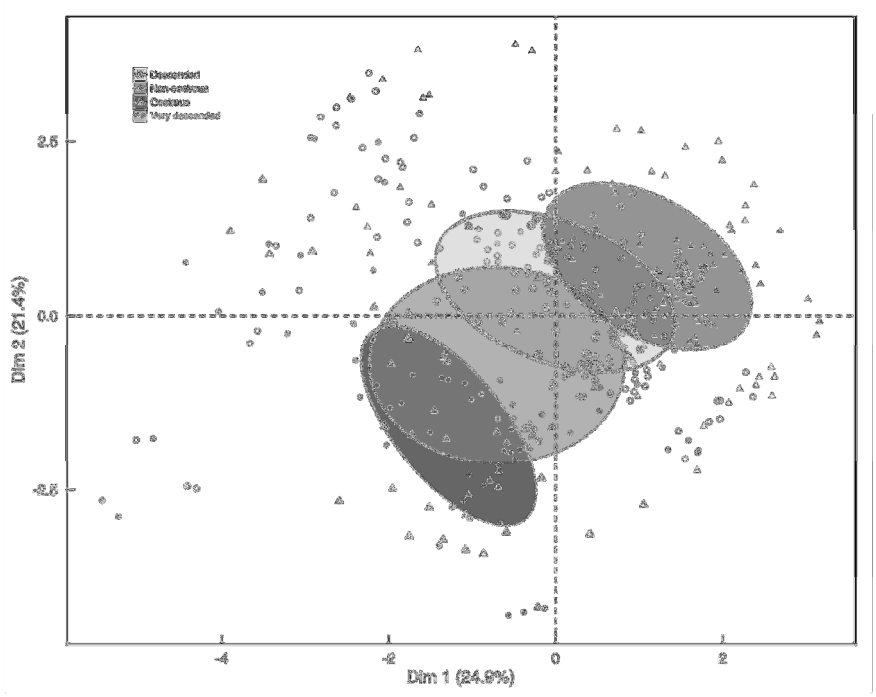


Figure 3 Factor map of *per diem* data projected in a reduced dimension space, defined by PC 1 (x-axis) and PC 2 (y-axis) of a principal component analysis. Ellipsoids depict the variance for each reproductive status, centered on respective means. Percentages represent the amount of variation in these data that were accounted for by the respective components (detailed in Table 2).

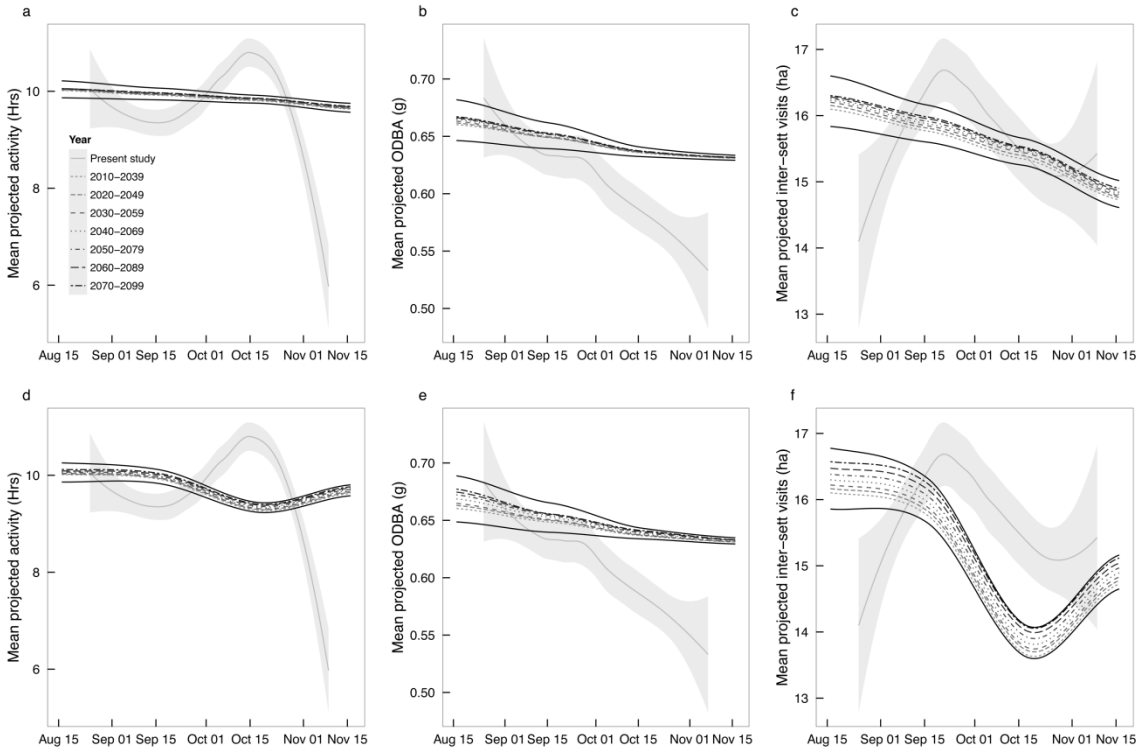


Figure 4 Mean projected *per diem* activity (Hrs); ODBA (*g*); and inter-sett visits (ha) under future climate projections over seven over-lapping time periods. Panels a – c depict projected behaviour under a low emissions scenario (IPCC SRES B1); d – f depict behaviour under a high emissions scenario (IPCC SRES A1F1). Trend lines depict Loess smoothed regressions. Shaded areas represent the standard error of the fit to behavioral data from the present study, whereas solid lines represent the error associated with projected responses.

A.7 **Supporting information**

Appendix S1 Further information relating to the calculation of the behavioral parameters 'ODBA', and 'activity'

In this appendix, we provide further information relating to the calculation of the behavioral parameter 'activity'. In this work we apply the threshold method developed by Noonan et al. (2014) to determine how active an animal was, on a

binary scale with 0 corresponding to inactive and 1 corresponding to active. This method was applied across all collared animals however, for clarity, we only present details for the badger wearing collar number 1.

Quantifying 'activity'

Approach

1. Acceleration was sampled at 8 Hz on 3 axes (i.e., heave; surge; and sway) simultaneously to provide a fine-scale record of animal behavior.

2. The vector measurements were transformed to observed dynamic body acceleration (ODBA; in g) as per the method in Wilson et al. (2006), here using a window length of 2 seconds to obtain the direction of g, followed by taking the L1 (absolute value) norm of the dynamic component.

3. ODBA values were then averaged over 1 minute bins, providing a metric of mean acceleration for each 1 minute time period. In this way, brief spikes in acceleration, which are due to knocks and bumps, are filtered out, leaving the trend itself. Applying this method, a total of 75,124 ODBA values were obtained for this badger over the 50 day sampling period. These values ranged from <0.01 to 11.19 g, with a mean value of 0.32 g ± 0.47 SD.

4. Mean ODBA values for each time period were then compared to a threshold value as per the method in Noonan et al. (2014) to determine if the animal was active (1) or inactive (0). Formally:

$$a\mathrm{k} = \mathrm{sgn}(\mathrm{ODBAk} - T) \qquad (1)$$

where ak [0:1] is the activity in the k^{th} window, sgn() is the signum function, ODBAk is the time averaged ODBA value and T is a threshold value.

The threshold value used in this study was 0.313. For badger 1, this resulted in 33.5% of ODBA values being categorised as 'active'; 66.5% as 'inactive'. Subsequently, this measure was used to calculate *per diem* activity (i.e., the duration of time per 24hr period that an individual was categorised as 'active'), as well as to investigate broad patterns in badgers' activity regimes (see below).

Activity and location

This binary activity measure was used to investigate an individual's willingness to engage in above-ground activity. This required a degree of certainty that the parameter 'activity' corresponded with the badger being aboveground. Although a total of 75,124 ODBA values were obtained for this badger, only 3,691 of these corresponded to a time when the badger was known to be within a monitored sett; 4,033 to a time when the badger was above-ground and within the detection range of a base-station. In contrast, 67,400 values corresponded to a time when the badger was at an unknown location either above-ground, or within an unmonitored sett. As such, limiting our analyses to ODBA values generated when the animal was at a known location would have resulted in a data loss of 89.7%.

For this reason, we make the assumption here that the majority of badger 'activity' occurs above-ground. Of the 3,691 ODBA values recorded while the badger was known to be within the sett, only 4.4% of these involved the badger being categorised as active. In contrast, of the 4,033 ODBA values recorded while

the badger was known to be above-ground, it was categorised as being active 82.9 % of the time. Of the ODBA values recorded when the badger was at an unknown location, 39.8 % of these were catagorised as periods of 'activity' (e.g., Fig. S1).

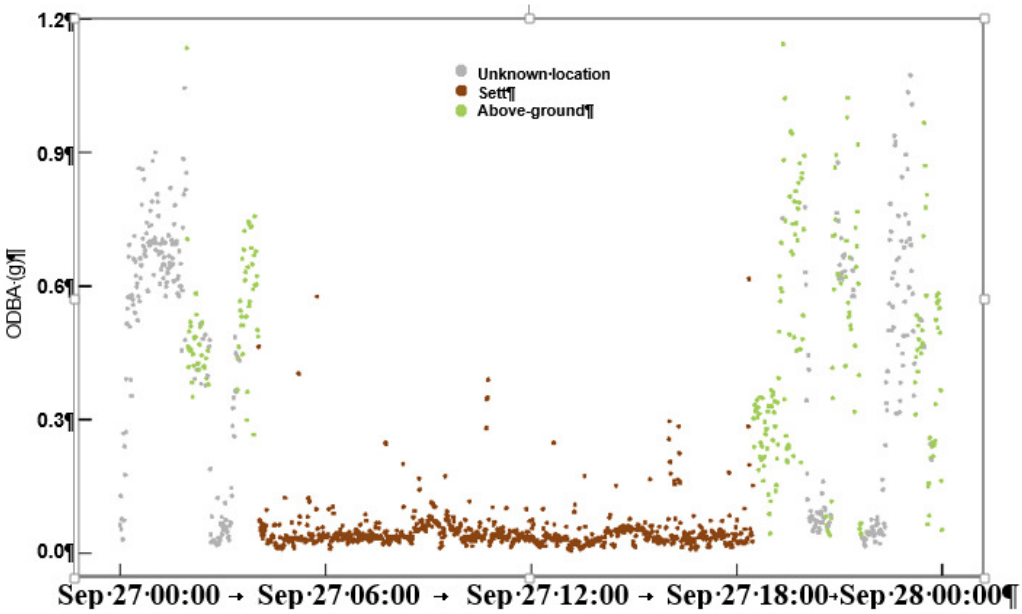


Figure S1: Scatterplot depicting ODBA values over the course of a 24 hour period. Colours indicate the individual's location categorised as i) sett (i.e., the badger was recorded within a monitored sett; ii) aboveground (i.e., above-ground and within the detection range of a base-station); and at an unknown location

.

Appendix S2 Analyses of trends in historical autumnal weather data (1853-2015) for the area surrounding the study site.

In this appendix, we provide analyses of trends in historical autumnal weather data (1853-2015). Meteorological records were obtained from the University of Oxford's Radcliffe Meteorological Station, located within 6 km from Wytham Woods and are available freely.

Temperature

Because of the sinusoidal nature of annual temperature fluctuations (Fig. S1a), we characterised autumnal temperatures in each year according to the trigonometric function:

$$T_m = \beta_0 + r_T \sin\left(\frac{2\pi}{\varphi} m\right)$$

Where β_0 represents the model intercept for each year; φ the phase (which was set to 12 such that each cycle has a period of one year); r_T the amplitude of temperature change; and $T_m$the mean temperature for calendar month m. To examine trends in the extent of seasonal temperature changes, we calculated r_T over the autumnal period (Sep – Dec) for each year.

There were significant positive trends in long-term mean daily maximum ($F_{[1,161]}$ = 48.20, $p < 0.001$, $R^2 = 0.23$; Fig. 2a), and minimum ($F_{[1,161]}$ = 58.11, $p < 0.001$, R^2 = 0.27; Fig. 2b) autumnal temperatures, equivalent to an increase of ca. 1.7 °C over the 162 year period. In contrast, we found negative, albeit non-significant trends in the

amplitude of seasonal maximum ($F_{[1,161]}$ = 2.39, p = 0.12, R^2 = 0.015; Fig. 2c), and minimum ($F_{[1,161]}$ = 2.02, p = 0.16, R^2 = 0.012; Fig. 2d) temperatures.

Rainfall

Rainfall did not exhibit any intra-annual trends (Fig. S2b). As such, we tested for linear trends in autumnal (Sep – Dec) rainfall patterns over time.

There was no significant trend in mean autumnal rainfall over the 162 year period ($F_{[1,161]}$ = 0.53, p = 0.47, R^2 = 0.003; Fig. 2e).

Figure S1 Scatterplots depicting; a) sinusoidal functions based on mean daily maximum and minimum temperatures; and b) total monthly rainfall for the year 2010.

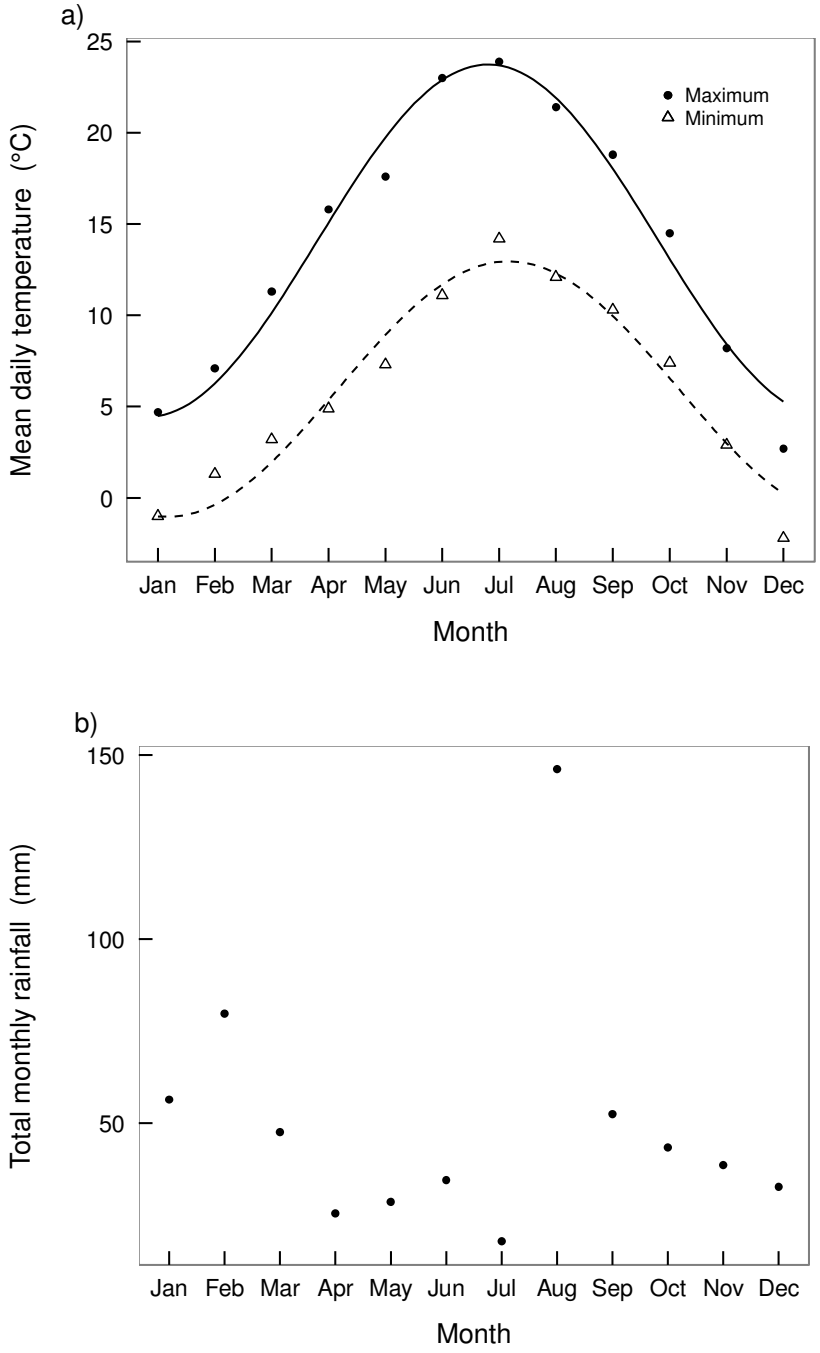
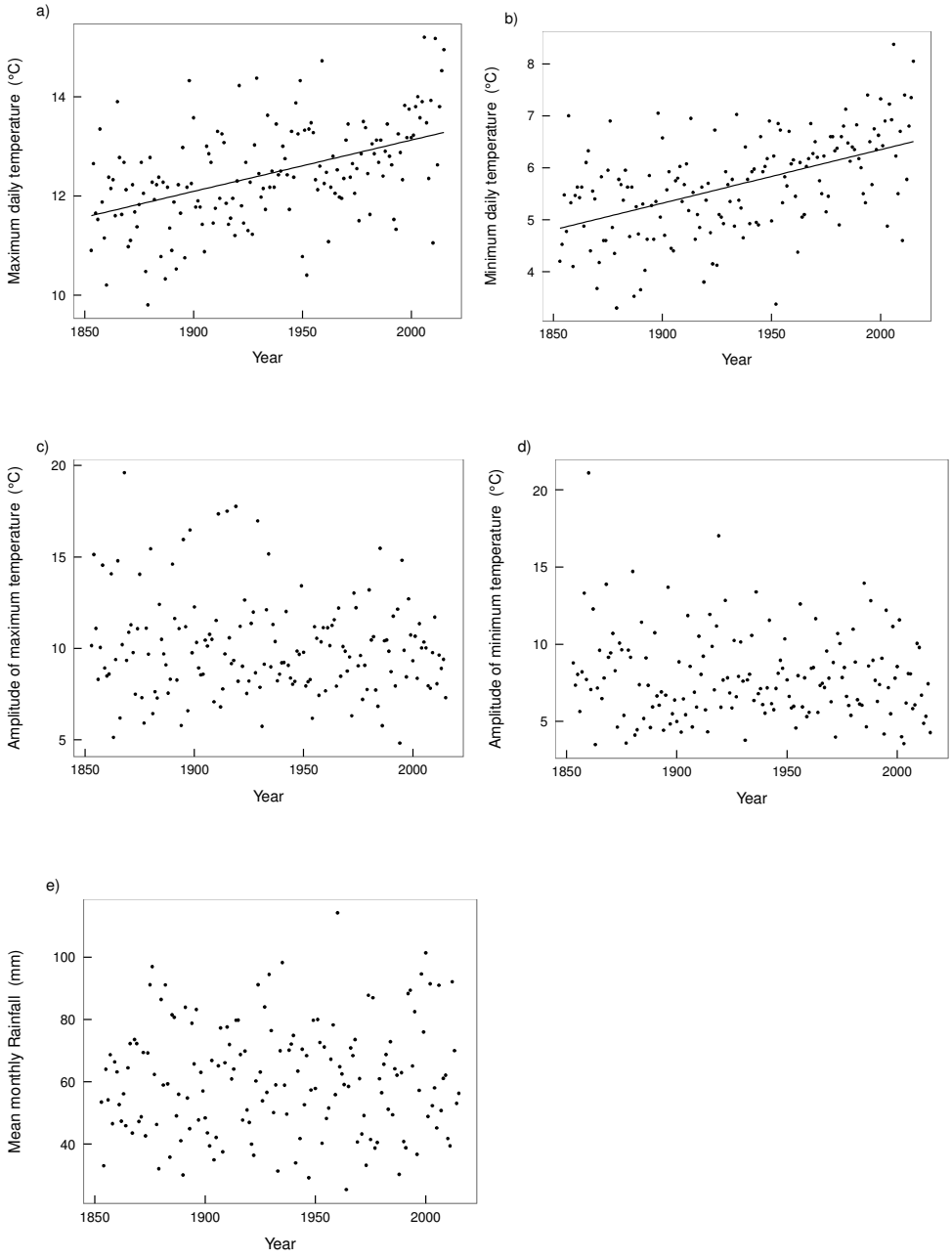


Figure S2 Scatterplots depicting trends in weather parameters over time. Trend lines denote significant correlations (see details in text).



Appendix S3 Analyses of the sett micro-climate over the course of the study period, establishing setts as thermal refugia.

In this appendix we provide analyses on the sett microclimate over the course of the study period, establishing setts as thermal refugia.

Sett entrance microclimate was measured at six of the nine sett sites monitored; three main setts (≥ 10 entrance holes); and three outlying setts (two entrance holes). Temperature and humidity data loggers (Lascar Electronics, Salisbury, UK), set at a 5 min sampling rate, were secured 1m inside of sett entrances. Although conditions within the sett differ asymptotically from above-ground conditions up to a depth of 3 m (Kaneko et al., 2010), a depth of 1 m was selected here as this represents the depth at which both main, and outlying setts begin to reach a thermal plateau. Thus, a tunnel depth of 1 m has sufficient thermal stability to provide an accurate estimate of deeper internal temperature. This depth and also represents the point inside the sett from which badgers consider the suitability of external weather when making their decisions on whether to leave the sett or not (Noonan et al., 2014; 2015a).

Due to main setts including more burrow entrances, data loggers were placed at two entrances, selected at random, versus a single data logger at outlying setts. Temperature and humidity records at main setts were then averaged between loggers. Data were collected continuously from September 2, 2014 to December 10, 2014, with a gap between October 8 to 14, 2014 when logger batteries were changed and the equipment was re-calibrated, generating a total of 26,817 data points per sett logger.

On a *per diem* basis, mean above-ground temperature did not differ significantly from temperatures 1m inside sett entrances at main setts (paired *t*-test: $t93 = -1.72$, $P =$

0.089), or outliers (paired *t*-test: $_{t93=}$ -0.74, $P = 0.46$). There was also no significant difference in temperature stability at a depth of 1m between outliers and main setts (Levene's: $F[1,187] = 0.30$, $P = 0.584$). Over time, however, sett temperatures were significantly more stable than above-ground temperatures (Levene's: $F[1,187] = 2.64$, $P < 0.005$; Table S1); where the proportion of time per diem that temperatures in sett entrances remained warmer than above-ground temperatures increased significantly over the autumnal period (sett: $r = 0.579$, $n = 94$, $P < 0.005$; outlier: $r = 0.613$, $n = 94$, $P < 0.005$; Fig. S1).

Table S1: Mean ± SD, and variance (CV) in temperature (°C), and humidity (RH) over the study period (2 September 2014 to 10 December 2014). Setts were sampled at a depth of 1m.

	Above-ground	Main sett	Outlying sett
Mean temp	10.7 ± 4.1	11.1 ± 2.5	11.0 ± 2.7
Temp CV	41.80%	22.70%	24.50%
Mean RH	96.8 ± 6.1	105.0 ± 1.8	103.5 ± 2.3
RH CV	7.50%	1.80%	2.50%

Mean per diem humidity was also significantly higher in both main setts and outliers than above-ground (paired t-test: t93 = -14.5, P <0.005; t93 = -13.6, P <0.005, respectively). As with temperature, at a 1m depth, relative humidity was significantly more stable in main and outlying setts than above-ground (Levene's: F[1,187] = 114.95, P <0.005; F[1,187] = 4.30, P = 0.040, respectively). Unlike temperature, sett conditions were consistently more humid than those above-ground, and therefore it was impossible to investigate any trends in proportional differences.

Implicitly, it cannot rain within setts, thus no comparison between conditions within the sett and above-ground was made.

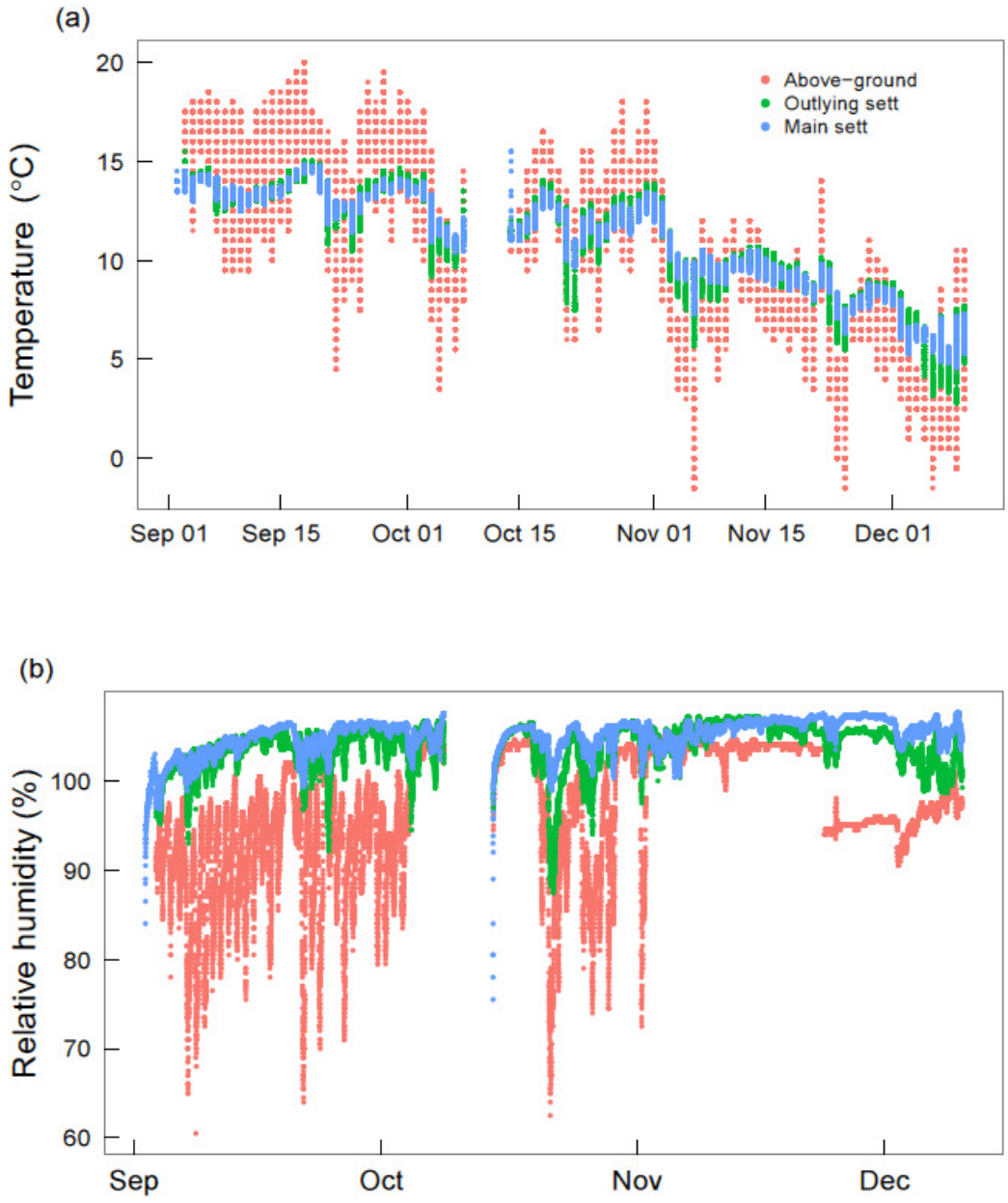


Figure S1: Scatter plots depicting a) temperatures, and b) relative humidity in main setts, outlying setts, and the above-ground environment over the 2014 study period (2 September to 10 December), sampled at 5 minute intervals. Main, and outlying setts were sampled at a depth of 1m.

Appendix S4 Details of predicted proportional changes in badger activity and energy expenditure in relation to climate projections under the IPCCs low (B1) and high (A1F1) emissions scenarios.

In this appendix, we provide details of predicted proportional changes in badger activity (Table S1) and energy expenditure (Table S2) in relation to climate projections under the IPCCs low emissions scenario SRES B1, and high emissions scenario SRES A1F1. The B1 scenario predicts an increase in atmospheric CO_2 to 500 to 600 ppm over the next century, resulting in a 1.1 to 2.9 °C rise in mean temperature and with no significant trends in precipitation. The A1F1 scenario predicts an increase in atmospheric CO_2 to 550 to 750 ppm over the next century, resulting in a 2.4 to 6.4 °C rise in mean temperature and a 0 to 5% decrease in relative humidity, with no significant trends in precipitation. These data exist as mean monthly values for seven overlapping time-periods; 2010 – 2039; 2020 – 2049; 2030 – 2059; 2040 – 2069; 2050 – 2079; 2060 – 2089; 2070 – 2099. Predictive models were parameterised based on the models presented in text.

Table S1: Proportional change in the duration (in hours) *of per diem* activity predicted under the IPCCs low emissions scenario SRES B1 climate projections for each time-period in comparison to *per diem* means exhibited by collared badgers in the present study. The standard deviation *(SD) of* means from the present study are also presented.

			Proportional Change						
Date	**Hrs**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-08-25	9.16	2.25	1.09	1.09	1.10	1.10	1.10	1.10	1.10
14-08-26	9.51	1.50	1.05	1.05	1.06	1.06	1.06	1.06	1.06
14-08-27	9.71	1.61	1.03	1.03	1.03	1.03	1.04	1.04	1.04
14-08-28	10.20	1.71	0.98	0.98	0.98	0.98	0.99	0.99	0.99
14-08-29	10.11	0.83	0.99	0.99	0.99	0.99	0.99	0.99	0.99
14-08-30	9.81	1.05	1.02	1.02	1.02	1.02	1.02	1.02	1.02
14-08-31	10.55	0.22	0.95	0.95	0.95	0.95	0.95	0.95	0.95
14-09-01	10.23	0.95	0.97	0.97	0.97	0.97	0.97	0.97	0.97
14-09-02	10.83	0.53	0.92	0.92	0.92	0.92	0.92	0.92	0.92
14-09-03	9.81	1.26	1.01	1.01	1.01	1.01	1.01	1.02	1.02
14-09-04	9.76	1.17	1.02	1.02	1.02	1.02	1.02	1.02	1.02
14-09-05	9.33	2.18	1.06	1.06	1.07	1.07	1.07	1.07	1.07
14-09-06	9.90	1.65	1.00	1.00	1.00	1.00	1.01	1.01	1.01
14-09-07	9.16	1.89	1.08	1.08	1.08	1.09	1.09	1.09	1.09
14-09-08	8.62	2.11	1.15	1.15	1.15	1.15	1.15	1.16	1.16
14-09-09	8.90	2.07	1.11	1.12	1.12	1.12	1.12	1.12	1.12
14-09-10	8.54	1.56	1.16	1.16	1.16	1.16	1.17	1.17	1.17
14-09-11	8.90	2.48	1.11	1.12	1.12	1.12	1.12	1.12	1.12
14-09-12	10.17	1.56	0.98	0.98	0.98	0.98	0.98	0.98	0.98
14-09-13	10.51	0.72	0.94	0.94	0.95	0.95	0.95	0.95	0.95
14-09-14	10.23	0.72	0.97	0.97	0.97	0.97	0.97	0.97	0.97
14-09-15	9.12	1.75	1.09	1.09	1.09	1.09	1.09	1.09	1.09
14-09-16	9.69	1.82	1.02	1.02	1.03	1.03	1.03	1.03	1.03
14-09-17	9.41	1.67	1.05	1.05	1.06	1.06	1.06	1.06	1.06
14-09-18	9.65	1.64	1.03	1.03	1.03	1.03	1.03	1.03	1.03
14-09-19	9.20	1.52	1.08	1.08	1.08	1.08	1.08	1.08	1.08

14-09-20	9.61	2.28	1.03	1.03	1.03	1.03	1.04	1.04	1.04
14-09-21	9.34	2.07	1.06	1.06	1.06	1.06	1.07	1.07	1.07
14-09-22	9.93	1.21	1.00	1.00	1.00	1.00	1.00	1.00	1.00
14-09-23	8.59	2.60	1.15	1.16	1.16	1.16	1.16	1.16	1.16

Table S1 –

			Proportional Change						
Date	**Hrs**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-09-24	8.92	1.71	1.11	1.11	1.11	1.11	1.12	1.12	1.12
14-09-25	9.56	2.36	1.04	1.04	1.04	1.04	1.04	1.04	1.04
14-09-26	10.04	1.71	0.99	0.99	0.99	0.99	0.99	0.99	0.99
14-09-27	8.33	1.67	1.19	1.19	1.19	1.19	1.19	1.20	1.20
14-09-28	9.60	2.05	1.03	1.03	1.03	1.04	1.04	1.04	1.04
14-09-29	10.35	1.19	0.96	0.96	0.96	0.96	0.96	0.96	0.96
14-09-30	10.32	1.73	0.96	0.96	0.96	0.96	0.96	0.97	0.97
14-10-01	8.66	1.83	1.13	1.13	1.14	1.14	1.14	1.14	1.14
14-10-02	10.23	2.02	0.96	0.96	0.96	0.96	0.96	0.96	0.96
14-10-03	10.44	0.85	0.94	0.94	0.94	0.94	0.94	0.94	0.94
14-10-04	10.56	1.45	0.93	0.93	0.93	0.93	0.93	0.93	0.93
14-10-05	11.21	1.68	0.88	0.88	0.88	0.88	0.88	0.88	0.88
14-10-06	10.31	1.27	0.95	0.95	0.95	0.95	0.96	0.96	0.96
14-10-07	10.33	1.15	0.95	0.95	0.95	0.95	0.95	0.95	0.95
14-10-08	10.83	1.69	0.91	0.91	0.91	0.91	0.91	0.91	0.91
14-10-09	10.56	0.89	0.93	0.93	0.93	0.93	0.93	0.93	0.93
14-10-10	10.93	1.15	0.90	0.90	0.90	0.90	0.90	0.90	0.90
14-10-11	10.25	1.98	0.96	0.96	0.96	0.96	0.96	0.96	0.96
14-10-12	11.14	1.67	0.88	0.88	0.88	0.88	0.88	0.88	0.88
14-10-13	9.64	1.12	1.02	1.02	1.02	1.02	1.02	1.02	1.02
14-10-14	10.66	0.91	0.92	0.92	0.92	0.92	0.92	0.92	0.92
14-10-15	10.41	1.38	0.94	0.94	0.94	0.95	0.95	0.95	0.95
14-10-16	10.93	0.93	0.90	0.90	0.90	0.90	0.90	0.90	0.90
14-10-17	10.61	1.31	0.92	0.93	0.93	0.93	0.93	0.93	0.93
14-10-18	11.07	1.03	0.89	0.89	0.89	0.89	0.89	0.89	0.89
14-10-19	10.26	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
14-10-20	10.25	1.36	0.96	0.96	0.96	0.96	0.96	0.96	0.96
14-10-21	9.45	2.19	1.04	1.04	1.04	1.04	1.04	1.04	1.04
14-10-22	10.65	1.75	0.92	0.92	0.92	0.92	0.92	0.92	0.93
14-10-23	11.70	1.21	0.84	0.84	0.84	0.84	0.84	0.84	0.84
14-10-24	10.59	1.86	0.93	0.93	0.93	0.93	0.93	0.93	0.93
14-10-25	11.16	1.34	0.88	0.88	0.88	0.88	0.88	0.88	0.88
14-10-26	10.85	1.30	0.90	0.91	0.91	0.91	0.91	0.91	0.91
14-10-27	10.89	1.51	0.90	0.90	0.90	0.90	0.90	0.90	0.91
14-10-28	11.12	1.00	0.88	0.88	0.88	0.88	0.89	0.89	0.89
14-10-29	9.86	0.96	0.99	1.00	1.00	1.00	1.00	1.00	1.00
14-10-30	11.15	0.92	0.88	0.88	0.88	0.88	0.88	0.88	0.88
14-10-31	10.99	1.42	0.89	0.89	0.90	0.90	0.90	0.90	0.90
14-11-01	10.42	1.94	0.92	0.93	0.93	0.93	0.93	0.93	0.93
14-11-02	9.67	1.03	1.00	1.00	1.00	1.00	1.00	1.00	1.00
14-11-03	4.85	3.16	1.99	1.99	1.99	1.99	2.00	2.00	2.00
14-11-04	5.79	4.75	1.66	1.67	1.67	1.67	1.67	1.67	1.67
14-11-05	5.57	2.79	1.73	1.73	1.73	1.73	1.74	1.74	1.74
14-11-06	9.18	2.21	1.05	1.05	1.05	1.05	1.05	1.05	1.06
14-11-07	4.32	6.10	2.23	2.23	2.24	2.24	2.24	2.24	2.25

Table S2: Proportional change in the duration (in hours) of *per diem* activity predicted under the IPCCs high emissions scenario SRES A1F1 climate projections for each time-period in comparison to *per diem* means exhibited by collared badgers in the present study. The standard deviation (SD) of means from the present study are also presented.

			Proportional Change						
Date	**Hrs**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-08-25	9.16	2.25	1.09	1.09	1.10	1.10	1.10	1.10	1.10
14-08-26	9.51	1.50	1.05	1.05	1.06	1.06	1.06	1.06	1.06
14-08-27	9.71	1.61	1.03	1.03	1.03	1.04	1.04	1.04	1.04
14-08-28	10.20	1.71	0.98	0.98	0.98	0.99	0.99	0.99	0.99
14-08-29	10.11	0.83	0.99	0.99	0.99	0.99	1.00	1.00	1.00
14-08-30	9.81	1.05	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-08-31	10.55	0.22	0.95	0.95	0.95	0.95	0.95	0.96	0.96
14-09-01	10.23	0.95	0.97	0.97	0.97	0.97	0.98	0.98	0.98
14-09-02	10.83	0.53	0.92	0.92	0.92	0.92	0.92	0.92	0.93
14-09-03	9.81	1.26	1.01	1.01	1.01	1.02	1.02	1.02	1.02

			Proportional Change						
Date	**Hrs**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-09-04	9.76	1.17	1.02	1.02	1.02	1.02	1.02	1.03	1.03
14-09-05	9.33	2.18	1.06	1.07	1.07	1.07	1.07	1.07	1.08
14-09-06	9.90	1.65	1.00	1.00	1.01	1.01	1.01	1.01	1.01
14-09-07	9.16	1.89	1.08	1.08	1.09	1.09	1.09	1.09	1.10
14-09-08	8.62	2.11	1.15	1.15	1.15	1.16	1.16	1.16	1.17
14-09-09	8.90	2.07	1.11	1.12	1.12	1.12	1.12	1.13	1.13
14-09-10	8.54	1.56	1.16	1.16	1.17	1.17	1.17	1.17	1.18
14-09-11	8.90	2.48	1.11	1.12	1.12	1.12	1.12	1.13	1.13
14-09-12	10.17	1.56	0.98	0.98	0.98	0.98	0.98	0.98	0.99
14-09-13	10.51	0.72	0.94	0.95	0.95	0.95	0.95	0.95	0.95
14-09-14	10.23	0.72	0.97	0.97	0.97	0.97	0.98	0.98	0.98
14-09-2	9.12	1.75	1.09	1.09	1.09	1.09	1.10	1.10	1.10

14-09-16	9.69	1.82	1.02	1.03	1.03	1.03	1.03	1.03	1.04
14-09-17	9.41	1.67	1.05	1.06	1.06	1.06	1.06	1.06	1.07
14-09-18	9.65	1.64	1.03	1.03	1.03	1.03	1.03	1.04	1.04
14-09-19	9.20	1.52	1.08	1.08	1.08	1.08	1.09	1.09	1.09
14-09-20	9.61	2.28	1.03	1.03	1.04	1.04	1.04	1.04	1.04
14-09-21	9.34	2.07	1.06	1.06	1.07	1.07	1.07	1.07	1.07
14-09-22	9.93	1.21	1.00	1.00	1.00	1.00	1.01	1.01	1.01
14-09-23	8.59	2.60	1.16	1.16	1.16	1.16	1.16	1.17	1.17
14-09-24	8.92	1.71	1.11	1.11	1.12	1.12	1.12	1.12	1.13
14-09-25	9.56	2.36	1.04	1.04	1.04	1.04	1.04	1.05	1.05
14-09-26	10.04	1.71	0.99	0.99	0.99	0.99	0.99	1.00	1.00
14-09-27	8.33	1.67	1.19	1.19	1.19	1.20	1.20	1.20	1.21
14-09-28	9.60	2.05	1.03	1.03	1.04	1.04	1.04	1.04	1.05
14-09-29	10.35	1.19	0.96	0.96	0.96	0.96	0.96	0.97	0.97
14-09-30	10.32	1.73	0.96	0.96	0.96	0.97	0.97	0.97	0.97
14-10-01	8.66	1.83	1.07	1.07	1.08	1.08	1.08	1.09	1.09
14-10-02	10.23	2.02	0.91	0.91	0.91	0.91	0.92	0.92	0.92
14-10-03	10.44	0.85	0.89	0.89	0.89	0.90	0.90	0.90	0.90
14-10-04	10.56	1.45	0.88	0.88	0.88	0.89	0.89	0.89	0.89
14-10-05	11.21	1.68	0.83	0.83	0.83	0.83	0.84	0.84	0.84
14-10-06	10.31	1.27	0.90	0.90	0.90	0.91	0.91	0.91	0.91
14-10-07	10.33	1.15	0.90	0.90	0.90	0.90	0.91	0.91	0.91
14-10-08	10.83	1.69	0.86	0.86	0.86	0.86	0.87	0.87	0.87
14-10-09	10.56	0.89	0.88	0.88	0.88	0.88	0.89	0.89	0.89
14-10-10	10.93	1.15	0.85	0.85	0.85	0.86	0.86	0.86	0.86
14-10-11	10.25	1.98	0.91	0.91	0.91	0.91	0.91	0.92	0.92
14-10-12	11.14	1.67	0.83	0.84	0.84	0.84	0.84	0.84	0.85
14-10-13	9.64	1.12	0.96	0.97	0.97	0.97	0.97	0.97	0.98
14-10-14	10.66	0.91	0.87	0.87	0.87	0.88	0.88	0.88	0.88
14-10-15	10.41	1.38	0.89	0.89	0.90	0.90	0.90	0.90	0.90
14-10-16	10.93	0.93	0.85	0.85	0.85	0.86	0.86	0.86	0.86
14-10-17	10.61	1.31	0.88	0.88	0.88	0.88	0.88	0.89	0.89
14-10-18	11.07	1.03	0.84	0.84	0.84	0.84	0.85	0.85	0.85
14-10-19	10.26	0.96	0.91	0.91	0.91	0.91	0.91	0.92	0.92
14-10-20	10.25	1.36	0.91	0.91	0.91	0.91	0.91	0.92	0.92
14-10-21	9.45	2.19	0.98	0.98	0.99	0.99	0.99	0.99	1.00
14-10-22	10.65	1.75	0.87	0.87	0.88	0.88	0.88	0.88	0.88
14-10-23	11.70	1.21	0.79	0.80	0.80	0.80	0.80	0.80	0.81
14-10-24	10.59	1.86	0.88	0.88	0.88	0.88	0.88	0.89	0.89
14-10-25	11.16	1.34	0.83	0.83	0.84	0.84	0.84	0.84	0.84
14-10-26	10.85	1.30	0.86	0.86	0.86	0.86	0.86	0.87	0.87
14-10-27	10.89	1.51	0.85	0.85	0.86	0.86	0.86	0.86	0.86
14-10-28	11.12	1.00	0.83	0.84	0.84	0.84	0.84	0.84	0.85
14-10-29	9.86	0.96	0.94	0.94	0.95	0.95	0.95	0.95	0.96
14-10-30	11.15	0.92	0.83	0.83	0.84	0.84	0.84	0.84	0.84
14-10-31	10.99	1.42	0.84	0.85	0.85	0.85	0.85	0.86	0.86
14-11-01	10.42	1.94	0.92	0.93	0.93	0.93	0.93	0.93	0.94
14-11-02	9.67	1.03	1.00	1.00	1.00	1.00	1.00	1.01	1.01
14-11-03	4.85	3.16	1.99	1.99	1.99	2.00	2.00	2.01	2.01
14-11-04	5.79	4.75	1.66	1.67	1.67	1.67	1.68	1.68	1.69
14-11-05	5.57	2.79	1.73	1.73	1.74	1.74	1.74	1.75	1.75
14-11-06	9.18	2.21	1.05	1.05	1.05	1.06	1.06	1.06	1.06
14-11-07	4.32	6.10	2.23	2.24	2.24	2.24	2.25	2.26	2.26

Table S3: Proportional change in *per diem* hourly ODBA (in *g*) expenditure predicted under the IPCCs low emissions scenario SRES B1 climate projections for each time-period in comparison to *per diem* means exhibited by collared badgers in the present study. The standard deviations (SD) of mean ODBA expenditure from the present study are also presented.

			Proportional Change						
Date	**ODBA**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-08-25	0.64	0.12	1.04	1.04	1.04	1.04	1.04	1.05	1.05
14-08-26	0.70	0.12	0.94	0.95	0.95	0.95	0.95	0.95	0.95
14-08-27	0.67	0.10	0.98	0.99	0.99	0.99	0.99	0.99	0.99
14-08-28	0.69	0.07	0.96	0.96	0.96	0.97	0.97	0.97	0.97
14-08-29	0.68	0.10	0.97	0.97	0.98	0.98	0.98	0.98	0.98
14-08-30	0.67	0.03	0.98	0.99	0.99	0.99	0.99	0.99	0.99
14-08-31	0.73	0.07	0.90	0.90	0.91	0.91	0.91	0.91	0.91
14-09-01	0.73	0.03	0.89	0.89	0.89	0.89	0.89	0.89	0.89
14-09-02	0.76	0.05	0.86	0.86	0.86	0.86	0.86	0.86	0.86
14-09-03	0.66	0.10	0.99	0.99	0.99	0.99	0.99	0.99	0.99
14-09-04	0.64	0.09	1.01	1.01	1.02	1.02	1.02	1.02	1.02
14-09-05	0.62	0.14	1.04	1.04	1.04	1.04	1.05	1.05	1.05
14-09-06	0.61	0.13	1.06	1.06	1.06	1.06	1.06	1.06	1.06
14-09-07	0.61	0.12	1.06	1.06	1.06	1.06	1.06	1.06	1.06
14-09-08	0.61	0.12	1.07	1.07	1.07	1.07	1.07	1.07	1.07
14-09-09	0.63	0.12	1.03	1.03	1.03	1.03	1.04	1.04	1.04
14-09-10	0.65	0.11	1.00	1.00	1.00	1.00	1.00	1.00	1.00
14-09-11	0.65	0.09	0.99	0.99	0.99	0.99	1.00	1.00	1.00
14-09-12	0.64	0.12	1.02	1.02	1.02	1.02	1.02	1.02	1.03
14-09-13	0.64	0.12	1.02	1.02	1.02	1.02	1.02	1.02	1.02
14-09-14	0.66	0.06	0.98	0.98	0.98	0.99	0.99	0.99	0.99
14-09-15	0.65	0.13	1.00	1.00	1.00	1.00	1.00	1.00	1.01
14-09-16	0.59	0.12	1.10	1.10	1.10	1.10	1.11	1.11	1.11
14-09-17	0.63	0.08	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-09-18	0.62	0.13	1.05	1.05	1.05	1.06	1.06	1.06	1.06
14-09-19	0.61	0.14	1.06	1.06	1.06	1.06	1.06	1.06	1.06
14-09-20	0.66	0.12	0.98	0.98	0.98	0.98	0.98	0.98	0.98
14-09-21	0.68	0.11	0.96	0.96	0.96	0.96	0.96	0.96	0.97

14-09-22	0.65	0.14	0.99	0.99	0.99	0.99	0.99	1.00	1.00
14-09-23	0.63	0.14	1.03	1.03	1.04	1.04	1.04	1.04	1.04
14-09-24	0.64	0.14	1.02	1.02	1.02	1.02	1.02	1.03	1.03
14-09-25	0.64	0.14	1.02	1.02	1.02	1.02	1.02	1.02	1.02
14-09-26	0.62	0.12	1.05	1.05	1.05	1.05	1.05	1.05	1.05
14-09-27	0.58	0.11	1.12	1.12	1.12	1.12	1.12	1.12	1.12
14-09-28	0.61	0.11	1.07	1.07	1.07	1.07	1.07	1.07	1.07
14-09-29	0.61	0.11	1.06	1.06	1.06	1.06	1.07	1.07	1.07
14-09-30	0.63	0.14	1.03	1.03	1.03	1.03	1.04	1.04	1.04
14-10-01	0.61	0.13	1.04	1.04	1.04	1.04	1.04	1.04	1.04
14-10-02	0.62	0.10	1.02	1.03	1.03	1.03	1.03	1.03	1.03
14-10-03	0.63	0.12	1.01	1.01	1.01	1.01	1.01	1.01	1.01
14-10-04	0.66	0.12	0.96	0.97	0.97	0.97	0.97	0.97	0.97
14-10-05	0.68	0.14	0.94	0.94	0.94	0.94	0.94	0.94	0.94
14-10-06	0.61	0.15	1.04	1.04	1.04	1.04	1.04	1.04	1.04
14-10-07	0.58	0.13	1.10	1.10	1.10	1.10	1.10	1.10	1.10
14-10-08	0.58	0.12	1.10	1.10	1.10	1.10	1.10	1.10	1.10
14-10-09	0.58	0.13	1.09	1.09	1.09	1.09	1.09	1.09	1.09
14-10-10	0.59	0.11	1.08	1.08	1.09	1.09	1.09	1.09	1.09
14-10-11	0.62	0.10	1.02	1.02	1.03	1.03	1.03	1.03	1.03
14-10-12	0.62	0.11	1.02	1.03	1.03	1.03	1.03	1.03	1.03
14-10-13	0.53	0.10	1.19	1.20	1.20	1.20	1.20	1.20	1.20
14-10-14	0.54	0.08	1.17	1.17	1.17	1.17	1.17	1.17	1.17
14-10-15	0.52	0.09	1.21	1.21	1.21	1.21	1.22	1.22	1.22
14-10-16	0.55	0.08	1.16	1.16	1.16	1.16	1.16	1.16	1.16
14-10-17	0.55	0.08	1.15	1.15	1.15	1.15	1.15	1.15	1.15
14-10-18	0.55	0.08	1.15	1.15	1.15	1.15	1.15	1.15	1.15
14-10-19	0.54	0.13	1.18	1.18	1.18	1.18	1.18	1.18	1.18
14-10-20	0.57	0.11	1.11	1.11	1.11	1.11	1.12	1.12	1.12
14-10-21	0.65	0.12	0.97	0.97	0.97	0.97	0.97	0.97	0.98
14-10-22	0.62	0.11	1.03	1.03	1.03	1.03	1.03	1.03	1.03
14-10-23	0.57	0.11	1.12	1.12	1.12	1.12	1.12	1.12	1.13
14-10-24	0.61	0.12	1.04	1.04	1.04	1.04	1.04	1.04	1.04

Table S3 – continued from previous page

			Proportional Change						
Date	**ODBA**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-10-25	0.60	0.13	1.06	1.06	1.06	1.06	1.06	1.06	1.06
14-10-26	0.61	0.15	1.04	1.04	1.04	1.04	1.04	1.04	1.04
14-10-27	0.61	0.17	1.04	1.04	1.04	1.04	1.04	1.04	1.05
14-10-28	0.53	0.09	1.20	1.20	1.20	1.20	1.20	1.20	1.20
14-10-29	0.50	0.12	1.26	1.26	1.27	1.27	1.27	1.27	1.27
14-10-30	0.51	0.12	1.24	1.24	1.24	1.24	1.24	1.24	1.24
14-10-31	0.51	0.09	1.25	1.25	1.25	1.25	1.26	1.26	1.26
14-11-01	0.56	0.10	1.13	1.13	1.13	1.13	1.13	1.13	1.13
14-11-02	0.53	0.11	1.18	1.18	1.18	1.18	1.18	1.18	1.18
14-11-03	0.56	0.20	1.12	1.12	1.12	1.12	1.12	1.12	1.12
14-11-04	0.50	0.15	1.26	1.26	1.26	1.26	1.26	1.26	1.26
14-11-05	0.56	0.20	1.12	1.12	1.12	1.12	1.13	1.13	1.13
14-11-06	0.48	0.09	1.30	1.30	1.30	1.30	1.30	1.30	1.30

Table S4: Proportional change in *per diem* hourly ODBA (in *g*) expenditure predicted under the IPCCs high emissions scenario SRES A1F1 climate projections for each time-period in comparison to *per diem* means exhibited by collared badgers in the present study. The standard deviations (SD) of mean ODBA expenditure from the present study are also presented.

			Proportional Change						
Date	**ODBA**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-08-25	0.64	0.12	1.04	1.04	1.04	1.05	1.05	1.06	1.06
14-08-26	0.70	0.12	0.94	0.94	0.94	0.96	0.96	0.96	0.97
14-08-27	0.67	0.10	0.98	0.98	0.98	1.00	1.00	1.00	1.01
14-08-28	0.69	0.07	0.96	0.96	0.96	0.97	0.98	0.98	0.98
14-08-29	0.68	0.10	0.97	0.97	0.97	0.99	0.99	0.99	1.00
14-08-30	0.67	0.03	0.98	0.98	0.98	1.00	1.00	1.00	1.01
14-08-31	0.73	0.07	0.90	0.90	0.90	0.92	0.92	0.92	0.93
14-09-01	0.73	0.03	0.89	0.89	0.89	0.89	0.90	0.90	0.90
14-09-02	0.76	0.05	0.86	0.86	0.86	0.86	0.87	0.87	0.87
14-09-03	0.66	0.10	0.98	0.99	0.99	0.99	0.99	0.99	1.00
14-09-04	0.64	0.09	1.01	1.02	1.02	1.02	1.02	1.02	1.03
14-09-05	0.62	0.14	1.04	1.04	1.05	1.05	1.05	1.05	1.05
14-09-06	0.61	0.13	1.06	1.06	1.06	1.06	1.07	1.07	1.07
14-09-07	0.61	0.12	1.06	1.06	1.06	1.06	1.07	1.07	1.07
14-09-08	0.61	0.12	1.07	1.07	1.07	1.07	1.08	1.08	1.08
14-09-09	0.63	0.12	1.03	1.03	1.04	1.04	1.04	1.04	1.04
14-09-10	0.65	0.11	1.00	1.00	1.00	1.00	1.01	1.01	1.01
14-09-11	0.65	0.09	0.99	0.99	1.00	1.00	1.00	1.00	1.00
14-09-12	0.64	0.12	1.02	1.02	1.02	1.03	1.03	1.03	1.03
14-09-13	0.64	0.12	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-09-14	0.66	0.06	0.98	0.99	0.99	0.99	0.99	0.99	0.99
14-09-15	0.65	0.13	1.00	1.00	1.00	1.01	1.01	1.01	1.01
14-09-16	0.59	0.12	1.10	1.10	1.11	1.11	1.11	1.11	1.11
14-09-17	0.63	0.08	1.02	1.02	1.03	1.03	1.03	1.03	1.03
14-09-18	0.62	0.13	1.05	1.06	1.06	1.06	1.06	1.06	1.07
14-09-19	0.61	0.14	1.05	1.06	1.06	1.06	1.06	1.07	1.07
14-09-20	0.66	0.12	0.98	0.98	0.98	0.98	0.99	0.99	0.99

14-09-21	0.68	0.11	0.96	0.96	0.96	0.97	0.97	0.97	0.97
14-09-22	0.65	0.14	0.99	0.99	0.99	1.00	1.00	1.00	1.00
14-09-23	0.63	0.14	1.03	1.04	1.04	1.04	1.04	1.05	1.05
14-09-24	0.64	0.14	1.02	1.02	1.02	1.03	1.03	1.03	1.03
14-09-25	0.64	0.14	1.02	1.02	1.02	1.02	1.02	1.03	1.03
14-09-26	0.62	0.12	1.05	1.05	1.05	1.05	1.06	1.06	1.06
14-09-27	0.58	0.11	1.12	1.12	1.12	1.12	1.13	1.13	1.13
14-09-28	0.61	0.11	1.06	1.07	1.07	1.07	1.07	1.08	1.08
14-09-29	0.61	0.11	1.06	1.06	1.07	1.07	1.07	1.07	1.07
14-09-30	0.63	0.14	1.03	1.03	1.03	1.04	1.04	1.04	1.04
14-10-01	0.61	0.13	1.04	1.04	1.04	1.04	1.04	1.04	1.05
14-10-02	0.62	0.10	1.03	1.03	1.03	1.03	1.03	1.03	1.03
14-10-03	0.63	0.12	1.01	1.01	1.01	1.02	1.02	1.02	1.02
14-10-04	0.66	0.12	0.97	0.97	0.97	0.97	0.97	0.97	0.97
14-10-05	0.68	0.14	0.94	0.94	0.94	0.94	0.94	0.94	0.95
14-10-06	0.61	0.15	1.04	1.04	1.04	1.05	1.05	1.05	1.05
14-10-07	0.58	0.13	1.10	1.10	1.10	1.10	1.10	1.11	1.11
14-10-08	0.58	0.12	1.10	1.10	1.10	1.10	1.11	1.11	1.11
14-10-09	0.58	0.13	1.09	1.09	1.09	1.10	1.10	1.10	1.10
14-10-10	0.59	0.11	1.09	1.09	1.09	1.09	1.09	1.09	1.09
14-10-11	0.62	0.10	1.03	1.03	1.03	1.03	1.03	1.03	1.03
14-10-12	0.62	0.11	1.03	1.03	1.03	1.03	1.03	1.03	1.03
14-10-13	0.53	0.10	1.20	1.20	1.20	1.20	1.20	1.20	1.20
14-10-14	0.54	0.08	1.17	1.17	1.17	1.17	1.17	1.18	1.18
14-10-15	0.52	0.09	1.21	1.22	1.22	1.22	1.22	1.22	1.22
14-10-16	0.55	0.08	1.16	1.16	1.16	1.16	1.16	1.16	1.17
14-10-17	0.55	0.08	1.15	1.15	1.15	1.16	1.16	1.16	1.16
14-10-18	0.55	0.08	1.15	1.15	1.15	1.15	1.16	1.16	1.16
14-10-19	0.54	0.13	1.18	1.18	1.18	1.18	1.18	1.18	1.19
14-10-20	0.57	0.11	1.11	1.12	1.12	1.12	1.12	1.12	1.12
14-10-21	0.65	0.12	0.97	0.98	0.98	0.98	0.98	0.98	0.98
14-10-22	0.62	0.11	1.03	1.03	1.03	1.04	1.04	1.04	1.04
14-10-23	0.57	0.11	1.12	1.13	1.13	1.13	1.13	1.13	1.13
14-10-24	0.61	0.12	1.04	1.04	1.04	1.05	1.05	1.05	1.05
14-10-25	0.60	0.13	1.06	1.06	1.06	1.06	1.06	1.06	1.07
14-10-26	0.61	0.15	1.04	1.04	1.04	1.04	1.04	1.04	1.05
14-10-27	0.61	0.17	1.04	1.05	1.05	1.05	1.05	1.05	1.05
14-10-28	0.53	0.09	1.20	1.20	1.20	1.20	1.21	1.21	1.21
14-10-29	0.50	0.12	1.27	1.27	1.27	1.27	1.27	1.27	1.28
14-10-30	0.51	0.12	1.24	1.24	1.24	1.24	1.24	1.25	1.25
14-10-31	0.51	0.09	1.25	1.26	1.26	1.26	1.26	1.26	1.26
14-11-01	0.56	0.10	1.13	1.13	1.13	1.13	1.13	1.13	1.13
14-11-02	0.53	0.11	1.18	1.18	1.18	1.18	1.18	1.18	1.18
14-11-03	0.56	0.20	1.12	1.12	1.12	1.12	1.12	1.13	1.13
14-11-04	0.50	0.15	1.26	1.26	1.26	1.26	1.26	1.26	1.26
14-11-05	0.56	0.20	1.12	1.12	1.13	1.13	1.13	1.13	1.13
14-11-06	0.48	0.09	1.30	1.30	1.30	1.31	1.31	1.31	1.31

			Proportional Change						
Date	**ODBA**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**

Table S5: Proportional change in *per diem* sett visits (ha) expenditure predicted under the IPCCs low emissions scenario SRES B1 climate projections for each time-period in comparison to *per diem* means exhibited by collared badgers in the present study. The standard deviations (SD) of mean inter-sett visits from the present study are also presented.

			Proportional Change						
Date	Sett **Visits**	**SD**	**2010 - 2039**	**2020 - 2049**	**2030 - 2059**	**2040 - 2069**	**2050 - 2079**	**2060 - 2089**	**2070 - 2099**
14-08-25	14.27	0.51	1.13	1.13	1.14	1.14	1.14	1.14	1.14
14-08-26	13.79	1.35	1.17	1.17	1.18	1.18	1.18	1.18	1.18
14-08-27	14.36	1.19	1.12	1.13	1.13	1.13	1.13	1.13	1.14
14-08-28	15.85	0.71	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-08-29	15.18	1.22	1.06	1.06	1.07	1.07	1.07	1.07	1.07
14-08-30	14.79	1.07	1.09	1.09	1.10	1.10	1.10	1.10	1.10
14-08-31	14.51	1.42	1.11	1.11	1.12	1.12	1.12	1.12	1.12
14-09-01	15.51	0.68	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-09-02	15.35	1.02	1.03	1.03	1.03	1.03	1.04	1.04	1.04
14-09-03	16.87	2.20	0.93	0.94	0.94	0.94	0.94	0.95	0.95
14-09-04	15.09	1.63	1.04	1.05	1.05	1.05	1.05	1.06	1.06
14-09-05	16.51	1.42	0.95	0.96	0.96	0.96	0.96	0.97	0.97
14-09-06	14.97	1.77	1.05	1.06	1.06	1.06	1.06	1.07	1.07
14-09-07	14.12	1.40	1.12	1.12	1.12	1.12	1.13	1.13	1.13
14-09-08	14.81	1.82	1.06	1.07	1.07	1.07	1.07	1.08	1.08
14-09-09	15.29	2.41	1.03	1.03	1.04	1.04	1.04	1.04	1.05
14-09-10	14.71	1.39	1.07	1.07	1.08	1.08	1.08	1.08	1.09
14-09-11	16.92	2.75	0.93	0.93	0.94	0.94	0.94	0.94	0.94
14-09-12	14.85	2.39	1.06	1.06	1.07	1.07	1.07	1.07	1.08
14-09-13	17.07	3.57	0.92	0.93	0.93	0.93	0.93	0.93	0.94
14-09-14	16.44	3.69	0.96	0.96	0.96	0.97	0.97	0.97	0.97

Table S5 – continued from previous page

Date	Sett Visits	SD	Proportional Change 2010 - 2039	2020 - 2049	2030 - 2059	2040 - 2069	2050 - 2079	2060 - 2089	2070 - 2099
14-09-15	16.95	3.53	0.93	0.93	0.93	0.94	0.94	0.94	0.94
14-09-16	17.93	4.02	0.88	0.88	0.88	0.89	0.89	0.89	0.89
14-09-17	15.50	2.99	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-09-18	18.57	3.82	0.85	0.85	0.85	0.86	0.86	0.86	0.86
14-09-19	16.81	3.76	0.94	0.94	0.94	0.94	0.95	0.95	0.95
14-09-20	17.83	4.48	0.88	0.89	0.89	0.89	0.89	0.89	0.90
14-09-21	14.77	3.23	1.07	1.07	1.07	1.07	1.08	1.08	1.08
14-09-22	15.41	2.69	1.02	1.03	1.03	1.03	1.03	1.04	1.04
14-09-23	14.06	2.37	1.12	1.12	1.13	1.13	1.13	1.13	1.14
14-09-24	17.22	4.68	0.92	0.92	0.92	0.92	0.92	0.93	0.93
14-09-25	17.20	3.27	0.92	0.92	0.92	0.92	0.93	0.93	0.93
14-09-26	18.04	4.88	0.87	0.88	0.88	0.88	0.88	0.88	0.89
14-09-27	16.27	2.94	0.97	0.97	0.97	0.98	0.98	0.98	0.98
14-09-28	16.87	2.16	0.93	0.94	0.94	0.94	0.94	0.95	0.95
14-09-29	15.52	2.48	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-09-30	16.97	4.33	0.93	0.93	0.93	0.94	0.94	0.94	0.94
14-10-01	17.46	4.27	0.88	0.88	0.88	0.89	0.89	0.89	0.89
14-10-02	15.27	1.95	1.01	1.01	1.01	1.01	1.01	1.02	1.02
14-10-03	17.00	4.00	0.90	0.91	0.91	0.91	0.91	0.91	0.91
14-10-04	14.73	2.09	1.04	1.05	1.05	1.05	1.05	1.05	1.05
14-10-05	15.50	2.04	0.99	0.99	1.00	1.00	1.00	1.00	1.00
14-10-06	15.80	3.27	0.97	0.97	0.98	0.98	0.98	0.98	0.98
14-10-07	15.03	2.44	1.02	1.02	1.03	1.03	1.03	1.03	1.03
14-10-08	15.97	2.13	0.96	0.96	0.97	0.97	0.97	0.97	0.97
14-10-09	16.01	3.08	0.96	0.96	0.96	0.97	0.97	0.97	0.97
14-10-10	15.22	1.31	1.01	1.01	1.01	1.02	1.02	1.02	1.02
14-10-11	15.70	2.18	0.98	0.98	0.98	0.99	0.99	0.99	0.99
14-10-12	16.39	2.54	0.94	0.94	0.94	0.94	0.95	0.95	0.95
14-10-13	14.87	2.13	1.03	1.04	1.04	1.04	1.04	1.04	1.04
14-10-14	16.57	3.57	0.93	0.93	0.93	0.93	0.94	0.94	0.94
14-10-15	16.72	5.11	0.92	0.92	0.92	0.93	0.93	0.93	0.93
14-10-16	15.63	2.65	0.98	0.98	0.99	0.99	0.99	0.99	0.99
14-10-17	15.05	3.13	1.02	1.02	1.03	1.03	1.03	1.03	1.03
14-10-18	14.44	1.34	1.06	1.07	1.07	1.07	1.07	1.07	1.08
14-10-19	16.08	2.07	0.95	0.96	0.96	0.96	0.96	0.96	0.97
14-10-20	15.00	0.84	1.02	1.03	1.03	1.03	1.03	1.03	1.04
14-10-21	15.68	2.41	0.98	0.98	0.99	0.99	0.99	0.99	0.99
14-10-22	15.26	1.69	1.01	1.01	1.01	1.01	1.02	1.02	1.02
14-10-23	14.17	1.19	1.08	1.09	1.09	1.09	1.09	1.09	1.10
14-10-24	15.58	2.56	0.99	0.99	0.99	0.99	0.99	1.00	1.00
14-10-25	13.63	1.23	1.13	1.13	1.13	1.14	1.14	1.14	1.14
14-10-26	15.07	1.96	1.02	1.02	1.03	1.03	1.03	1.03	1.03
14-10-27	14.15	1.71	1.08	1.09	1.09	1.09	1.09	1.10	1.10
14-10-28	14.60	2.31	1.05	1.05	1.06	1.06	1.06	1.06	1.06
14-10-29	14.49	1.62	1.06	1.06	1.07	1.07	1.07	1.07	1.07
14-10-30	14.63	1.63	1.05	1.05	1.06	1.06	1.06	1.06	1.06
14-10-31	15.09	1.95	1.02	1.02	1.02	1.03	1.03	1.03	1.03
14-11-01	13.38	0.84	1.10	1.10	1.11	1.11	1.11	1.11	1.11
14-11-02	15.04	2.62	0.98	0.98	0.98	0.98	0.99	0.99	0.99
14-11-03	14.34	1.33	1.03	1.03	1.03	1.03	1.03	1.04	1.04
14-11-04	14.47	2.43	1.02	1.02	1.02	1.02	1.03	1.03	1.03
14-11-05	14.18	0.74	1.04	1.04	1.04	1.04	1.05	1.05	1.05
14-11-06	16.99	3.69	0.87	0.87	0.87	0.87	0.87	0.87	0.88

Table S6: Proportional change in *per diem* sett visits (ha) expenditure predicted under the IPCCs high emissions scenario SRES A1F1 climate projections for each time-period in comparison to *per diem* means exhibited by collared badgers in the present study. The standard deviations (SD) of mean inter-sett visits from the present study are also presented.

Date	Sett Visits SD		Proportional Change 2010 - 2039	2020 - 2049	2030 - 2059	2040 - 2069	2050 - 2079	2060 - 2089	2070 - 2099
14-08-25	14.27	0.51	1.13	1.13	1.14	1.14	1.15	1.15	1.16
14-08-26	13.79	1.35	1.17	1.17	1.18	1.18	1.19	1.19	1.20

Date	Sett Visits	SD	Proportional Change 2010 - 2039	2020 - 2049	2030 - 2059	2040 - 2069	2050 - 2079	2060 - 2089	2070 - 2099
14-08-27	14.36	1.19	1.12	1.13	1.13	1.14	1.14	1.15	1.15
14-08-28	15.85	0.71	1.02	1.02	1.02	1.03	1.03	1.04	1.05
14-08-29	15.18	1.22	1.06	1.06	1.07	1.07	1.08	1.09	1.09
14-08-30	14.79	1.07	1.09	1.09	1.10	1.10	1.11	1.11	1.12
14-08-31	14.51	1.42	1.11	1.11	1.12	1.12	1.13	1.14	1.14
14-09-01	15.51	0.68	1.02	1.02	1.02	1.03	1.03	1.04	1.05
14-09-02	15.35	1.02	1.03	1.03	1.03	1.04	1.05	1.05	1.06
14-09-03	16.87	2.20	0.93	0.94	0.94	0.95	0.95	0.96	0.96
14-09-04	15.09	1.63	1.04	1.05	1.05	1.06	1.06	1.07	1.08
14-09-05	16.51	1.42	0.95	0.96	0.96	0.97	0.97	0.98	0.98
14-09-06	14.97	1.77	1.05	1.06	1.06	1.07	1.07	1.08	1.09
14-09-07	14.12	1.40	1.12	1.12	1.13	1.13	1.14	1.14	1.15

14-09-08	14.81	1.82	1.06	1.07	1.07	1.08	1.08	1.09	1.10
14-09-09	15.29	2.41	1.03	1.03	1.04	1.04	1.05	1.06	1.06
14-09-10	14.71	1.39	1.07	1.08	1.08	1.09	1.09	1.10	1.10
14-09-11	16.92	2.75	0.93	0.94	0.94	0.94	0.95	0.95	0.96
14-09-12	14.85	2.39	1.06	1.07	1.07	1.07	1.08	1.09	1.09
14-09-13	17.07	3.57	0.92	0.93	0.93	0.94	0.94	0.95	0.95
14-09-14	16.44	3.69	0.96	0.96	0.97	0.97	0.98	0.98	0.99
14-09-15	16.95	3.53	0.93	0.93	0.94	0.94	0.95	0.95	0.96
14-09-16	17.93	4.02	0.88	0.88	0.89	0.89	0.90	0.90	0.91
14-09-17	15.50	2.99	1.02	1.02	1.03	1.03	1.04	1.04	1.05
14-09-18	18.57	3.82	0.85	0.85	0.86	0.86	0.86	0.87	0.87
14-09-19	16.81	3.76	0.94	0.94	0.94	0.95	0.95	0.96	0.97
14-09-20	17.83	4.48	0.88	0.89	0.89	0.90	0.90	0.91	0.91
14-09-21	14.77	3.23	1.07	1.07	1.08	1.08	1.09	1.09	1.10
14-09-22	15.41	2.69	1.02	1.03	1.03	1.04	1.04	1.05	1.05
14-09-23	14.06	2.37	1.12	1.13	1.13	1.14	1.14	1.15	1.16
14-09-24	17.22	4.68	0.92	0.92	0.92	0.93	0.93	0.94	0.94
14-09-25	17.20	3.27	0.92	0.92	0.92	0.93	0.93	0.94	0.94
14-09-26	18.04	4.88	0.87	0.88	0.88	0.89	0.89	0.90	0.90
14-09-27	16.27	2.94	0.97	0.97	0.98	0.98	0.99	0.99	1.00
14-09-28	16.87	2.16	0.93	0.94	0.94	0.95	0.95	0.96	0.96
14-09-29	15.52	2.48	1.02	1.02	1.02	1.03	1.03	1.04	1.05
14-09-30	16.97	4.33	0.93	0.93	0.94	0.94	0.95	0.95	0.96
14-10-01	17.46	4.27	0.78	0.79	0.79	0.79	0.80	0.81	0.81
14-10-02	15.27	1.95	0.90	0.90	0.90	0.91	0.91	0.92	0.92
14-10-03	17.00	4.00	0.81	0.81	0.81	0.82	0.82	0.83	0.83
14-10-04	14.73	2.09	0.93	0.93	0.94	0.94	0.95	0.95	0.96
14-10-05	15.50	2.04	0.88	0.89	0.89	0.90	0.90	0.91	0.91
14-10-06	15.80	3.27	0.87	0.87	0.87	0.88	0.88	0.89	0.89
14-10-07	15.03	2.44	0.91	0.92	0.92	0.92	0.93	0.94	0.94
14-10-08	15.97	2.13	0.86	0.86	0.86	0.87	0.87	0.88	0.88
14-10-09	16.01	3.08	0.86	0.86	0.86	0.87	0.87	0.88	0.88
14-10-10	15.22	1.31	0.90	0.90	0.91	0.91	0.92	0.92	0.93
14-10-11	15.70	2.18	0.87	0.88	0.88	0.88	0.89	0.90	0.90
14-10-12	16.39	2.54	0.84	0.84	0.84	0.85	0.85	0.86	0.86
14-10-13	14.87	2.13	0.92	0.93	0.93	0.93	0.94	0.95	0.95
14-10-14	16.57	3.57	0.83	0.83	0.83	0.84	0.84	0.85	0.85
14-10-15	16.72	5.11	0.82	0.82	0.83	0.83	0.84	0.84	0.84
14-10-16	15.63	2.65	0.88	0.88	0.88	0.89	0.89	0.90	0.90
14-10-17	15.05	3.13	0.91	0.91	0.92	0.92	0.93	0.93	0.94
14-10-18	14.44	1.34	0.95	0.95	0.96	0.96	0.97	0.97	0.98
14-10-19	16.08	2.07	0.85	0.86	0.86	0.86	0.87	0.87	0.88
14-10-20	15.00	0.84	0.91	0.92	0.92	0.92	0.93	0.94	0.94
14-10-21	15.68	2.41	0.87	0.88	0.88	0.89	0.89	0.90	0.90
14-10-22	15.26	1.69	0.90	0.90	0.91	0.91	0.92	0.92	0.93
14-10-23	14.17	1.19	0.97	0.97	0.97	0.98	0.99	0.99	1.00
14-10-24	15.58	2.56	0.88	0.88	0.89	0.89	0.90	0.90	0.91
14-10-25	13.63	1.23	1.00	1.01	1.01	1.02	1.02	1.03	1.04
14-10-26	15.07	1.96	0.91	0.91	0.92	0.92	0.93	0.93	0.94
14-10-27	14.15	1.71	0.97	0.97	0.98	0.98	0.99	0.99	1.00
14-10-28	14.60	2.31	0.94	0.94	0.95	0.95	0.96	0.96	0.97
14-10-29	14.49	1.62	0.95	0.95	0.95	0.96	0.96	0.97	0.97
14-10-30	14.63	1.63	0.94	0.94	0.94	0.95	0.95	0.96	0.97
14-10-31	15.09	1.95	0.91	0.91	0.92	0.92	0.93	0.93	0.94
14-11-01	13.38	0.84	1.10	1.10	1.11	1.11	1.12	1.12	1.13
14-11-02	15.04	2.62	0.98	0.98	0.99	0.99	0.99	1.00	1.00
14-11-03	14.34	1.33	1.03	1.03	1.03	1.04	1.04	1.05	1.05
14-11-04	14.47	2.43	1.02	1.02	1.02	1.03	1.03	1.04	1.04
14-11-05	14.18	0.74	1.04	1.04	1.05	1.05	1.06	1.06	1.07
14-11-06	16.99	3.69	0.87	0.87	0.87	0.88	0.88	0.89	0.89

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