The mechanisms of senescence in wild European badgers

Submitted by:

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To the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

October 2014

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Overwhelming evidence for senescence, the within-individual decline in performance at advanced age, has now been documented in the natural populations of many taxa. As such, the focus of senescence research is shifting from simply documenting its existence, towards understanding the fundamental mechanisms underpinning it and determining which environmental factors give rise to the considerable variation in senescence rates observed in nature. In this thesis I use a wild population of European badgers (*Meles meles*) to investigate three important traits implicated in, or arising as a direct product of, senescence; immune cell telomere length, pro-inflammatory cytokine response and body mass declines in late life. My work reveals rare longitudinal evidence for the existence of senescence in immune traits in a wild mammal. First, I show that within-individual declines in immune cell telomere length occur with increasing age (Chapter 2). Second, after demonstrating that immune cell telomere length displays repeatable between-individual differences in adulthood, I show that the environmental conditions experienced in early-life contribute to such between-individual variation. Individuals that experienced harsh early-life environmental conditions had shorter immune cell telomere lengths than those that experienced favourable conditions (Chapter 3). Third, I show that within-individual declines in a second immune trait, pro-inflammatory cytokine response, also occur with age (Chapter 4). However, the declines in immune cell telomere length and pro-inflammatory cytokine response occur independently of one another (Chapter 4). Finally I take advantage of a 37 year longitudinal dataset to reveal that sex differences in body mass senescence arise as a consequence of the scale of intra-sexual competition experienced in early adulthood (Chapter 5). Taken together this work provides novel evidence suggesting that age-related declines in immunocompetence can contribute to whole organism senescence in the wild. Furthermore, evidence that early life environmental and social conditions can markedly influence senescence rates has important implications for our understanding of the drivers of variation in senescence rates observed within natural populations.

Acknowledgements

I want to start by thanking to my supervisors Andrew Young and Dez Delahay for their constant help and support throughout my PhD. Andy's unwavering passion for 'the question' and his ability to think deep about pretty much any subject you throw at him, has been a constant source of inspiration for me! Aside from many a fruitful discussion, Dez came to the rescue with an incredibly well-timed seedcorn grant just when my hopes of successfully funding the lab work were beginning to fade. I couldn't have asked to have worked with two nicer blokes over the last three years.

Huge thanks to staff at the National Wildlife Management Centre Animal & Plant Health Agency / Animal and Veterinary Health Laboratories Agency / Food and Environment Research Agency (or whatever else they are renamed to in the next six months) for performing all the fieldwork, bTB diagnostic testing and maintenance of the long term dataset. Working at the Centre for Ecology and Conservation has been a real pleasure. The academics, post-grads, techs, support staff and students (far too many good people in all categories to mention by name) are all great. It is a special place. Massive thanks go to Team Telomere (Michelle Hares and Emma Wood) for help with qPCR assay development, it was a struggle but we got there!

Huge love and thanks to my friends (who, given that you are still reading this, I can probably count in their number), my family and Laura for being there when it counts (and forgiving me when I am not). To the residents of 289 (plus Jenny, Dom and Faye) and the wonderful Iain Stott, I have enjoyed every minute of living with you all! Whilst the meat Sundays, bacon cakes, snackadiums, Lurpak, double chocolate digestives, gravy doughnuts, boozy nights in Falmouth and their resultant aggressive cereal-based incidents have certainly shortened my lifespan, it has been a blast. Finally, let it be known that the final score for the 'MHCB CEC Invitational 8-Ball Pool Match of Champions' is as follows: Chris Beirne – 44, Mikey Hawkes 46. Well done Mikey, after I lead for so long I can't believe you fluked it at the death!

Table of Contents

List of Tables

Chapter 1

Chapter 2:

Chapter 3

Chapter 4

Chapter 5

List of Figures

Authors Declaration

All trapping records, bait-marking, bovine tuberculosis testing and blood sampling was performed and compiled by Food and Environment Research Agency (now Animal Health and Veterinary Laboratories Agency) staff. My supervisors commented on earlier drafts of this work. Additional comments on drafts were sought from Mike Cant, Alastair Wilson and Andy Russell for Chapter 5. With these exceptions, I declare that the work contained in this thesis is my own and has not been submitted for any other degree or award. All badger trapping and biological sample collection was carried out under license from the United Kingdom Home Office (license number PPL60/3609) according to the Animals (Scientific Procedures) Act 1986.

Publications

At the time of printing the following chapters have been published or are under review exactly as they appear here:

Chapter 2: Published: PlosOne

Age-related declines and disease-associated variation in immune cell telomere length in a wild mammal

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Chapter 5: Submitted: Proceedings B

Sex differences in senescence in the European badger: the role of intra-sexual competition in early adulthood

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Chapter 1: General introduction

Organismal senescence, or ageing, is the cumulative and intrinsic changes that occur within an individual over time and lead to the impairment of performance in late life and, ultimately, death. One of the reasons why biologists have been fascinated by senescence was captured succinctly by Williams (1957):

"It is indeed remarkable that after a seemingly miraculous feat of morphogenesis a complex metazoan should be unable to perform the much simpler task of merely maintaining what is already formed"

This observation is particularly pertinent as senescence is seemingly not inevitable for all forms of life, and considerable variation in longevity both within and between species exists in nature: from gastrotichs which have a lifespan of just a few days, to the quaking aspen (*Populus tremuloides*) which is estimated to live over 10,000 years (Vaupel *et al.* 2004). Given the extreme natural variation in longevity it is perhaps surprising that for many years the study of senescence was dominated by biomedical research conducted on humans and genetically inbred laboratory model organisms. Indeed, many researchers were sceptical that senescence even occurred in the wild as they thought extrinisic mortality would kill individuals before they reached the ages at which senescence could be detected (Nussey *et al.* 2013). Biomedical research has been, and remains, vital in furthering our understanding of senescence. However, it has become increasingly clear that if a deeper understanding of the evolutionary ecology of senescence is to be reached, researchers need to expand their focus to a broader taxonomic scale operating under natural environmental conditions (Monaghan *et al.* 2008).

Senescence in the wild

Despite the initial scepticism, empirical evidence that senescence occurs in wild animals is now overwhelming. A recent review found evidence of senescence in 340 studies of 175 different animal species (Nussey *et al.* 2013). Given the prevalence of senescence in the wild, the field is moving away from simply documenting its existence, towards attempts to

understand the mechanisms that underpin it and why such marked variation in its age of onset and rate of progression are observed in nature. Initial studies on senescence in natural populations focused on age-related changes in survival probability and reproductive output as these are key determinants of individual fitness (e.g. Bérubé *et al.* 1999; Loison *et al.* 1999). More recently, studies have emerged examining age-related changes in other phenotypic traits such as telomere length (e.g. Monaghan & Haussmann 2006), immunocompetence (e.g. Palacios *et al.* 2007) and body mass (e.g. Tafani *et al.* 2012). Such traits are interesting as they can exhibit age-related declines themselves, but may also be part of the mechanism that gives rise to age-related declines in reproductive success and survival.

Theories of Senescence

To understand the processes driving variation in senescence rates, we must first consider the theories hypothesised to explain its existence. Senescence limits an organism's reproductive potential, and thus there should be strong selection acting against its persistence. There are currently three prevailing theories explaining the existence of senescence (Kirkwood & Austad 2000). First, the 'mutation accumulation theory' posits that senescence is a by-product of the accumulation of deleterious mutations that act in late life to reduce the survival and reproductive success of old individuals (Medawar 1952). Such deleterious mutations acting in late life are less likely to be removed from the genome by natural selection than those that act in early life because the force of selection weakens with age as fewer individuals survive to advanced ages. This idea was extended by Williams (1957) into the 'antagonistic pleiotropy theory'. Here, genes that confer positive effects in early life are favoured by natural selection even if they have deleterious effects in late life. As the contribution of a given gene to fitness is determined by the size of its effect multiplied by the probability of an individual surviving to a given age to experience that effect, genes with small beneficial effects in early-life may be selected for even if they result in large deleterious effects in late life. Finally, the 'disposable soma theory' developed by Kirkwood (1977) states that whilst effective somatic maintenance is required to survive, maintenance mechanisms (such as DNA repair or oxidative stress protection) are likely to be metabolically costly. When resources are limited, trade-offs between investment in somatic maintenance and investment in other costly activities (e.g. growth and reproduction) are

therefore predicted to occur. Senescence therefore results from the age-related accumulation of unrepaired damage to somatic cells and tissues.

The three theories are not necessarily mutually exclusive, they could all be operating to greater or lesser extents in different species (Monaghan *et al.* 2008) and there appears to be at least some evidence for all three (Kirkwood & Austad 2000). Mutation accumulation remains the most controversial of the three, with only weak evidence for its existence restricted to laboratory experiments on *Drosophila melanogaster* (e.g Service *et al.* 1988). The evidence base for the antagonistic pleiotropy and disposable soma theories are much stronger and extend to natural populations living under ecologically realistic conditions. Evidence for the disposable soma theory of ageing typically involves observations that reproductive investment in early life increasing the rate of senescence in late life (e.g. Bouwhuis *et al.* 2010; Hammers *et al.* 2013). Charmantier *et al.* (2006) showed that a free ranging population of Mute swans (*Cygnus olor*) were subject to a evolutionary trade-off consistent with antagonistic pleiotropy: the rate of senescence was faster in individuals who started reproducing earlier and, crucially, these traits were heritable. It is beyond the scope of this thesis to determine which theory best fits the currently available evidence of senescence. That said, the antagonistic pleiotropy and disposable soma theories (which can also be termed 'life history theories of senescence') have the wider scope to explain the inter-individual variation in senescence rates so often observed in nature (Nussey *et al.* 2013). The life history theories posit that the rate at which an individual senesces is dependent upon where each individual lies on the trade-off continuum between somatic maintenance (in the case of disposable soma) or protective gene expression (in the case of antagonistic pleiotropy) and investment in other costly traits. It is possible to test these predictions using laboratory experiments to manipulate such trade-offs, however it is difficult to determine to what extent the results are influenced by the study organisms unique (usually inbred) genetic background or the laboratory conditions in which they are reared. Consequently, contributions from longitudinal analyses of natural populations living in ecologically realistic environmental conditions will be invaluable to understanding the factors generating natural variation in senescence rates (Monaghan *et al.* 2008).

Telomeres

One candidate mechanism underpinning late-life declines in performance is age-related declines in telomere length (Monaghan 2010). Telomeres are protective nucleoprotein complexes found at the end of all eukaryotic chromosomes. At the cellular level, telomeres are essential for chromosome stability, protecting from loss of protein-coding DNA during cell replication and damage owing to aberrant DNA repair mechanisms (Blackburn 1991). In vertebrate somatic cells, telomere length typically shortens with each successive cell division, at least in cells that do not express the telomere restoration enzyme telomerase (or other restoration mechanisms). Telomere shortening is largely due to the incomplete replication of telomeres during cell replication (the 'end replication problem'; Blackburn 1991) and oxidative damage (von Zglinicki 2002). When a cell's telomeres reach a critically short threshold it stops dividing and either undergoes programmed cell death or enters a senescent state (Campisi 2003). It is thought that the accumulation of such 'senescent cells' with age can reduce the replicative capacity and metabolic function of the tissues in which they reside, which in turn contributes to the ageing phenotype of the whole organism (Sahin *et al.* 2011). Given that telomere loss can result in cellular senescence, and the accumulation of such senescent cells with age is thought to contribute to whole organism senescence, why do all cells not simply express the enzyme telomerase to maintain their telomeres? Cancerous cells are known to up-regulate the enzyme telomerase or possess dysfunctional telomeres, allowing them to escape cellular senescence and proliferate rapidly (Campisi *et al.* 2001). Thus, one potential explanation for the tight control of telomerase expression is that cellular senescence acts as a mechanism to protect organisms from cancer (Campisi 2001).

Scaling up to the whole organism level, studies from humans, laboratory model organisms and domesticated animals have been highly informative in understanding the implications of telomere dynamics for health and lifespan. Telomere length has been found to be positively correlated with the life spans of different dog breeds (Fick *et al.* 2012), and telomere length in early life predicts lifespan within a captive population of zebra finches (Heidinger *et al.* 2012). Progressive telomere shortening has been shown to be associated

with a host of human diseases including premature ageing syndromes (Blasco 2005), inflammatory disease (Kong, Lee & Wang 2013) and depression (Wolkowitz *et al.* 2011). Epel *et al.* (2004) were the first to link 'life stress' to telomere attrition, whereby females with higher perceived stress indexes had shorter telomeres. The link between stress and telomere attrition has since been replicated in a variety of controlled environment studies on other organisms (e.g. Kotrschal *et al.* 2007; Sohn *et al.* 2012).

Interest in telomere dynamics within the field of ecology and evolution has stemmed from the possibility that they may reflect an individual's previous 'life stress' and therefore facilitate measuring the trade-offs associated with life-history theory (Salomons *et al.* 2009; Monaghan 2014). So whilst controlled environment studies have been highly informative, it has become increasingly apparent that studies of the telomere dynamics of natural populations are required in order to understand the role that environmental effects may play in modulating telomere dynamics (Haussmann & Marchetto 2010). The vast majority of what we know about the natural telomere dynamics of wild populations stems from avian studies. Consistent with previously reported links to longevity, average telomere length declines with age in many of the wild bird species tested thus far (e.g. Juola *et al.* 2006; Bize *et al.* 2009; Young *et al.* 2013 but see Haussmann *et al.* 2003) and short telomeres have been associated with increased mortality (e.g. Barrett *et al.* 2012). By contrast, studies of the natural telomere dynamics of wild mammals are almost non-existent (Table 1). Furthermore, despite a strong biomedical literature linking disease state with telomere length (Fossel 2012), such a link has yet to be probed in wild vertebrate populations. The unifying feature linking all biomedical, controlled environment and ecological studies of telomeres to date is the substantial variation observed in telomere length among individuals of the same age. The primary challenge at present is elucidating the genetic and environmental factors that generate such variation and determining their implications for individual life history trajectories.

Table 1. The taxonomic scope of research on mammalian telomere dynamics to date. Just one study to date has examined telomere lengths in a wild mammal, and aside from biomedical research no studies have investigated the link between disease and telomere dynamics. Where multiple publications exist the publication is proceeded by 'e.g.' and a representative reference cited. 'Wild?' denotes whether or not the organisms in questions where from wild populations living under natural environmental conditions (*'s denote wild caught individuals reared and infected under laboratory conditions), 'Disease?' denotes where links between telomere dynamics and infection/disease states were assessed and 'Age?' denotes whether or not the effects of age were accounted for.

Species	Publication	Wild?	Disease?	Age?
Humans (*H. sapiens*)	(e.g. Njajou *et al.* 2007)	-	Yes	Yes
Cynologus macaque (*M. fascicularis*)	(e.g. Lee *et al.* 2002)	No	No	Yes
Hamadryas Baboon (*P. hamadryas*)	(e.g. Baerlocher *et al.* 2007)	No	No	Yes
House Mouse (*M. musculus musculus*)	(e.g. Ilmonen *et al.* 2008)	Yes*	Yes	No
Brown Rat (*R. norvegicus*)	(e.g. Cherif *et al.* 2003)	No	No	Yes
Algerian Mouse (*M. spretus*)	(Coviello & Prowse 1997)	No	No	Yes
Edible Dormouse (*G. glis*)	(Turbill *et al.* 2013)	No	No	Yes
Dog (*C. lupus domesticus*)	(e.g. Fick *et al.* 2012)	No	No	Yes
Domestic Pigs (*S. scrofa domesticus*)	(Jiang *et al.* 2004)	No	No	Yes
Dairy Cattle (*B. taurus*)	(Brown *et al.* 2012)	No	No	Yes
Djungarian hamster (*P. sungorus*)	(Turbill *et al.* 2012)	No	No	Yes
Australian Sea Lion (*N. cinerea*)	(Izzo *et al.* 2011)	Yes	No	Yes

Immunity

Biomedical research on humans and laboratory model organisms has also implicated immunosenescence, declining immune system function with age, in causing increased susceptibility to disease and mortality at advanced ages (Larbi *et al.* 2008). As such, immunosenescence could be a key factor underpinning senescent declines in survival

probabilities in natural populations. There is increasing recognition of the importance of examining the causes and implications of variation in immune phenotypes within natural populations (Martin, Weil & Nelson 2006; Hawley & Altizer 2011). However, compelling evidence of immunosenescence in wild vertebrate populations remains rare, particularly in mammals (Maizels & Nussey 2013). Evidence for immunosenescence in the wild has been largely restricted to two studies on mammals and a handful of studies in birds and reptiles (Table 2). The evidence base for immunosenescence in the wild (Table 2) can be summarised with three general points: *i*) some evidence exists for declines in the acquired cell-mediated immune response with age, *ii)* conflicting evidence exists for declines in the acquired or innate humoral component and *iii)* there is a marked taxonomic bias in the studies conducted to date towards birds and reptiles. In mammals, Nussey *et al.* (2012) found cross-sectional evidence for changes consistent with immunosenescence in several immune parameters of free-living Soay sheep (declines in CD4+ and γδ T-cells and increases in baseline inflammatory markers). To date, the vast majority of research examining immunosenescence in the wild has been cross-sectional in approach (Table 2). Cross-sectional analyses make it impossible to determine if the changes observed arise through within-individual age-related changes in immune parameters, or population level processes (e.g. cohort effects or age-dependent selective appearance or disappearance of individuals from the monitored population). In the only longitudinal assessment of wild vertebrate immunosenescence to date, Schneeberger *et al.* (2014), found evidence of within-individual declines in the numbers of circulating immune cells in the Greater sac-winged bat (*Saccopteryx bilineata*), but no within-individual change in baseline Immunoglobulin G concentration or plasma bactericidal capacity. The scarcity of such evidence from wild populations makes the generality of the findings of immunosenescence research on laboratory model organisms and humans difficult to determine (Maizels & Nussey 2013). Consequently, whether the patterns of immunosenescence observed under standardised laboratory conditions translate to free living vertebrates in natural environments remains largely unknown.

Table 2. Evidence for immunosenescence in wild vertebrate populations. An updated version of the table that appeared in Palacios et al. (2011) with evidence from captive populations removed and subsequent publications added. Where; 'Senescence?' = evidence of senescence; 'longitudinal' = studies following the same individuals through time (* = longitudinal sampling occurred but within-individual changes not addressed); IgG = Immunoglobulin G; shaded areas = mammalian studies. References: [a]Haussmann *et al.* (2005), [b]Palacios *et al.* (2007), [c]Nussey *et al.* (2012), [d]Saino *et al.* (2003), [e]Cichon *et al.* (2003), [f]Ujvari (2005), [g]Apanius & Nisbet (2003), [h]Zimmerman *et al.* (2013), [i]Schneeberger *et al.* (2014), [j]Møller & Haussy (2007), [k]Sparkman & Palacios (2009)

Immune component, technique used, and species	Senescence?	Longitudinal?
Acquired cell-mediated immune response		
In vivo skin swelling response		
Storm Petrel (*Oceanodroma leucorhoa*)[a]	Yes	No
Tree Swallow (*Tachycineta bicolor*)[a]	Yes	No
T-cell proliferation response		
Tree Swallow[b]	Yes	No
Circulating T-cell Subsets		
Soay Sheep (*Ovis aries*)[c]	Yes	No
Acquired humoral immune component (B-cell function)		
In vivo antibody production in response to antigenic challenge		
Barn Swallow (*Hirundo rustica*)[d]	Yes	No
Collared Flycatcher (*Ficedula albicollis*)[e]	Yes	No
Water python (*Liasis fuscus*)[f]	Yes	No
Tree Swallow [b]	No	No
In vitro B-cell proliferation in response		
Tree Swallow [b]	No	No
Total IgG level in plasma		
Common Tern (*Sterna hirundo*)[g]	No	No*
Red-eared slider turtle (*Trachemys scripta*)[h]	No	No
Greater sac-winged bat (*Saccopteryx bilineata*)[i]	No	Yes
Innate humoral immune component		
Natural antibody level in plasma		
Barn Swallow[j]	Yes	No
Tree Swallow[b]	No	No
Garter snake (*Thamnophis elegans*)[k]	No	No
Red-eared slider turtle[h]	No	No
Complement-mediated lysis		
Barn Swallow[j]	Yes	No
Tree Swallow[b]	No	No
Garter snake [k]	No	No
Greater sac-winged bat[i]	No	Yes
Adaptive and Innate Immunity		
White blood cell counts		
Greater sac-winged bat[i]	Yes	Yes

This thesis investigates the patterns and mechanistic underpinnings of senescence in the wild population of European Badgers (*Meles meles*) at Woodchester Park, UK. European badgers (Figure 1) are omnivorous principally nocturnal members of the mustelid family that live in underground setts, or dens. They are facultatively social and form large groups with clearly demarked territories where population densities are high (Carpenter *et al.* 2005), as is the case at Woodchester Park. This badger population lends itself well to studying senescence in the wild for several reasons. First, both male and female badgers are already known to undergo reproductive senescence: male reproductive output declines steeply from five years of age whereas female reproductive output declines less steeply from three (Dugdale *et al.* 2011b). Second, marked individuals of known age are repeatedly captured throughout their lives giving good temporal resolution in the traits measured and facilitating longitudinal, within-individual analyses. Third, the resident badger population has been the subject of continuous trapping and epidemiological monitoring since 1977 (Delahay *et al.* 2000), which has resulted in a remarkable longitudinal dataset comprising of over 15,000 captures of over 2,500 unique individuals, the majority of which are of known age. This facilitates modelling of key traits (e.g. body mass) across an individual's whole lifespan. Finally, this dataset is almost unique in terms of the comprehensive bovine tuberculosis testing that is undertaken on all animals at every capture, which gives a near unparalleled insight into the role of immunity, infection and disease with age in a wild mammalian population.

The mating system of the European badger is polygynandrous. Breeding occurs annually with females usually giving birth in and around February in the UK. Females have an average litter size of two (range 1-5). Cubs typically take six to eight weeks to wean and twelve to fifteen months to become sexually mature. Reproductive activity peaks during a post-partum oestrous, although there may be a secondary mating peak in late summer (Cresswell *et al.* 1992; Buesching, Heistermann & Macdonald 2009). Mating and pregnancy are decoupled as females exhibit delayed implantation of fertilised blastocysts. While mating may occur asynchronously between early spring and late autumn, females become approximately synchronously pregnant during the winter (Roper 2010). In high density

populations there are multiple breeding females and males within each social group. Significant skew in reproductive success has been detected among females within the same social group (Dugdale, Macdonald & Pope 2008). Rates of extra-group paternity are high (50%) in the European badger, which may act as a mechanism of inbreeding avoidance given that dispersal is limited and within-group relatedness is high (Carpenter *et al.* 2005; Annavi *et al.* 2014).

Figure 1: Two European badgers foraging. By Mark Robinson [CC-BY-2.0 (http://creativecommons.org/licenses/by/2.0)]

Study site and methods

The core study site comprises of ~11km^2 of mixed deciduous and coniferous woodland surrounded by farmland (Figure 2A) (Delahay, Walker & Forrester 2006b). The badger population size fluctuates between 200 to 350 individuals (Delahay *et al.* 2013). Each year, the boundaries of all social group territories were approximated by bait marking in spring (Figure 2B) (Delahay *et al.* 2000), and badgers were trapped at all active setts for two nights, four times per year. Cubs trapped prior to independence (twelve to fifteen weeks old) and/or before they had passed a minimum weight threshold (2kg) were immediately released. All other badgers captured were anaesthetised and identified through a unique tattoo administered at first capture. A range of variables were recorded for each capture, including capture location, sex, body mass (to nearest 100g), body length (to the nearest cm), body condition, tooth wear score and age class (juveniles < 1 year, adult ≥ 1). Additionally, samples of blood, saliva, urine, lung aspirate and wound swabs were taken for

bovine tuberculosis diagnostic testing. Precise dates of birth or ages of individuals cannot be readily determined as birth occurs underground on an unknown date. Throughout this thesis I calculate all ages from the 20th of February of an individual's first year of life, as this reflects the peak period for badger births in the UK (Roper 2010).

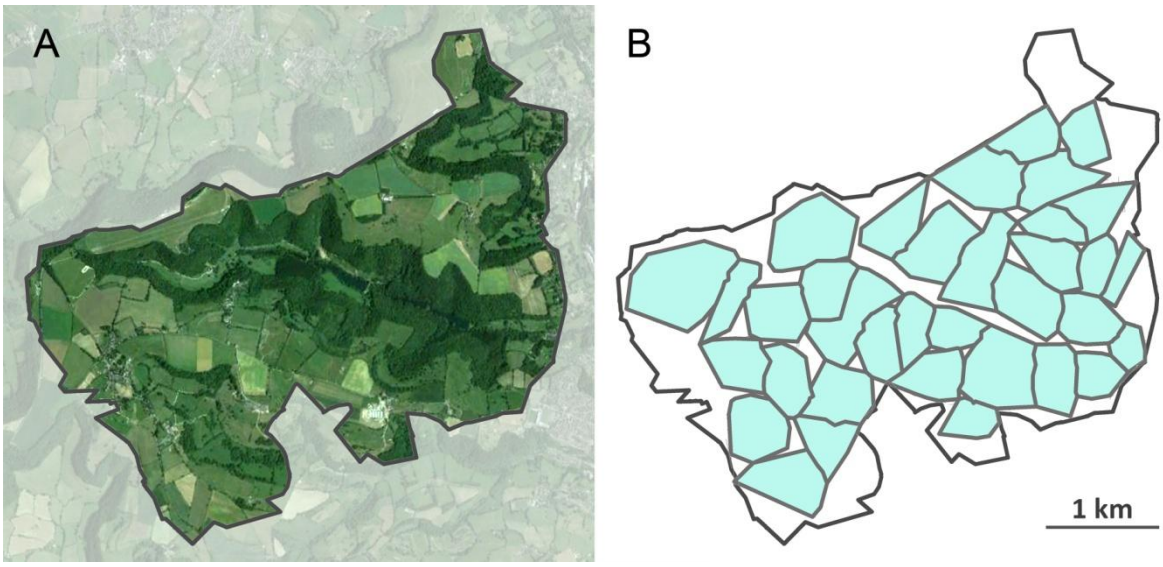


Figure 2: Study site and social groups. The extent of the Woodchester Park study site and a representation of the territories of the focal social groups (from 1994).

Bovine tuberculosis

The Woodchester Park project was set up to study the natural maintenance and transmission of the bacterium *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB). Badgers have long been suspected to be a wildlife reservoir for bTB, which has lead to repeated attempts to reduce badger populations in the UK and Ireland in order to reduce the prevalence of the disease in cattle (Roper 2010). Despite the lack of management within the Woodchester Park population, a recent estimate of long-term disease prevalence suggested that bTB prevalence was relatively low, with roughly 10-20% of the badgers captured each year testing as 'exposed' to bTB and 5-10% of individuals actively disseminating the infection (Delahay *et al.* 2013). However, prevalence is likely to have been underestimated in this study owing to the use of historical bTB diagnostic assays with low sensitivity. Since 1982, bTB testing has involved a combination of a serological ELISA assessing the presence of bTB antibodies in blood (Brock Test; see Goodger *et al.* 1994) and the microbiological culture of clinical samples (i.e. sputum, faeces, urine and

wound or abscess swabs). The sensitivity of Brocks test is 40-53% and the sensitivity of microbiological culture ~20% (Chambers *et al.* 2009), which means individuals infected with bTB were frequently mis-diagnosed as disease negative. In recent years the utility of the Brock Test has been questioned and so it has be largely superseded by the introduction of two more-sensitive assays: i) the Interferon-gamma (IFNγ) assay (an enzyme immunoassay assessing lymphocyte responsiveness to bTB; sensitivity = 71%) and ii) the Brock StatPak assay (a lateral-flow immunoassay assessing the presence of bTB antibodies; sensitivity = 58-80%). Despite the recent addition of these more sensitive assays, designating individuals as bTB 'positive' or 'negative' remains challenging. Throughout the course of this thesis I use a one-way progressive system whereby individuals can transition to elevated disease classifications when appropriate tests are positive, but can never return to lower classifications (as in Delahay *et al.* 2013; Tomlinson *et al.* 2013). In Chapters 2-4 I use the outcomes of the IFNγ assay, StatPak assay and microbiological culture to classify diseased individuals. In Chapter 5, which utilises more historical data than the previous chapters, I use the outcomes of the Brock Test and microbiological culture as, while they have their limitations (Drewe *et al.* 2010), this information is available for the vast majority of the captures in the longitudinal database.

Thesis Aims and Outline

In this thesis I use the European badger (*Meles meles*) as a model species to examine how important physiological traits change with age (immune cell telomere length, pro-inflammatory cytokine response and body mass) and, where appropriate, explore what factors might influence individual variation in the rate at which they change. Specifically, the aims of this thesis are to: *i)* explore the age and disease-related changes in immune cell telomere length; *ii)* examine putative early-life factors giving rise to inter-individual variation in adult telomere length, *iii)* investigate age and disease-related variation in the pro-inflammatory cytokine response and *iv)* examine the causes of sex differences in body mass senescence rates. I deal with each briefly below:

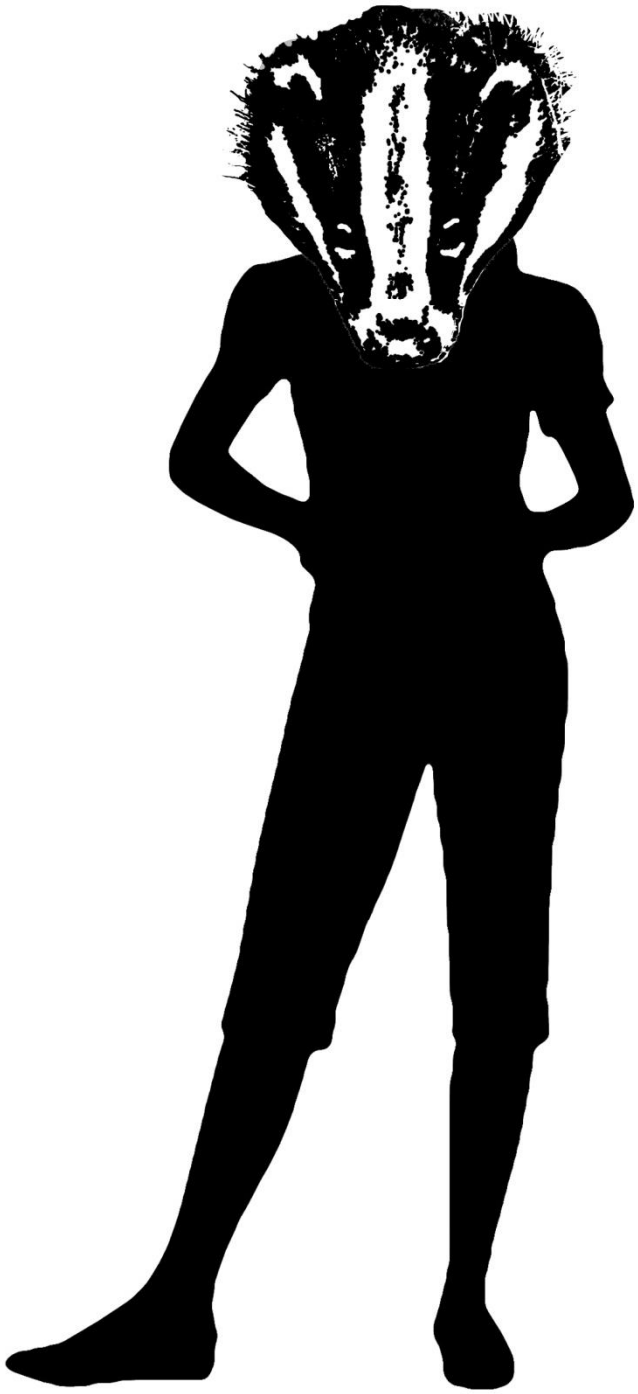
Chapter 2 provides a comprehensive assessment of within-individual age-related changes in immune cell telomere length in a wild vertebrate. This chapter also highlights associations between an individual's current disease status and their immune cell telomere length.

Chapter 3 investigates the early-life factors that influence the marked inter-individual variation in immune cell telomere lengths observed in adulthood. I find strong evidence that the environmental conditions experienced in the first year of life play a role in determining adult immune cell telomere length, independently of the rate at which individuals grow.

Chapter 4 investigates the age-related changes in the pro-inflammatory cytokine response, using interferon-gamma, an important pro-inflammatory signalling molecule. This represents the first longitudinal assessment of within-individual changes in immune signalling in a wild population. The chapter also explores the putative link between immune cell telomere length and the pro-inflammatory immune response.

Chapter 5 utilises the full scope of the 37 year longitudinal dataset to provide the first test of the hypothesis that sex differences in senescence are a product of somatic maintenance costs arising from sex differences in the intensity of intra-sexual competition experienced in early adulthood.

Finally, Chapter 6 gives a general consideration of the work contained within this thesis.



Chapter 2: Age-related declines and disease-associated variation in immune cell telomere length in a wild mammal

Abstract

Immunosenescence, the deterioration of immune system capability with age, may play a key role in mediating age-related declines in whole-organism performance, though the mechanisms that underpin immunosenescence are poorly understood. Biomedical research on humans and laboratory models has documented age and disease related declines in the telomere lengths of leukocytes ('immune cells'), stimulating interest in their having a potentially general role in the emergence of immunosenescent phenotypes. However, it is unknown whether such observations generalise to the immune cell populations of wild vertebrates living under ecologically realistic conditions. Here we examine longitudinal changes in the mean telomere lengths of immune cells in wild European badgers (*Meles meles*). Our findings provide the first evidence of within-individual age-related declines in immune cell telomere lengths in a wild vertebrate. That the rate of age-related decline in telomere length appears to be steeper within individuals than at the overall population level raises the possibility that individuals with short immune cell telomeres and/or higher rates of immune cell telomere attrition may be selectively lost from this population. We also report evidence suggestive of associations between immune cell telomere length and bovine tuberculosis infection status, with individuals detected at the most advanced stage of infection tending to have shorter immune cell telomeres than disease positive individuals. While male European badgers are larger and show higher rates of annual mortality than females, we found no evidence of a sex difference in either mean telomere length or the average rate of within-individual telomere attrition with age. Our findings lend support to the view that age-related declines in the telomere lengths of immune cells may provide one potentially general mechanism underpinning age-related declines in immunocompetence in natural populations.

Immunosenescence is the gradual deterioration of immune system capability with age, and may be a major factor influencing age-related declines in whole-organism performance (Larbi *et al.* 2008). However, the physiological mechanisms that underpin immunosenescence are poorly understood. Biomedical studies have revealed age-related declines in the average length of immune cell telomeres (protective nucleoprotein complexes found at the ends of eukaryotic chromosomes) in humans and laboratory models (Haussmann *et al.* 2003; Vera *et al.* 2012; Armanios 2013), and indicate that immune cell telomere lengths can act as biomarkers of age-related disease (Fossel 2012). This research has led to the suggestion that age-related declines in immune cell telomere lengths may contribute to the immunosenescence-related health declines previously documented in humans and wild vertebrates (Weng 2012). Studies of wild birds have now frequently documented age-related declines in the telomere lengths of nucleated erythrocytes (red blood cells; Haussmann *et al.* 2003; Salomons *et al.* 2009; Barrett *et al.* 2012b), which share the same haematopoietic stem cell precursors as immune cells. However, despite growing evidence of immunosenescence in wild vertebrate populations (Palacios *et al.* 2011), it has yet to be investigated whether such age-related declines in telomere length also occur in the immune cell populations of wild vertebrates.

Telomeres consist in part of repetitive sequences of DNA $(TTAGGG)^n$ that often decrease in length over time (largely due to oxidative damage and the end replication problem during cell division; Monaghan & Haussmann 2006) and may trigger cellular senescence once they become critically short (Weng 2012). While the enzyme telomerase can recover telomere length, telomerase expression appears to be suppressed in the somatic cells of many mammals, due perhaps to a role for telomere attrition in tumor suppression mechanisms (Flores, Benetti & Blasco 2006). Age-related declines in the telomere lengths of immune cells *per se* may be expected to arise as (i) many immune cells are constantly renewed via the repeated division of stem cell precursors and (ii) cells of the adaptive immune system (B- and T-lymphocytes) undergo rapid proliferation in response to invading pathogens (Goronzy, Fujii & Weyand 2006). As critically short telomeres can compromise the function of haematopoietic stem cells (the cells that produce immune cells and erythrocytes; Flores

et al. 2006) and reduce the efficacy of immune cell responses (e.g. lymphocyte proliferation potential; Effros 2011), age-related declines in immune cell telomere length may act to reduce immunocompetence in older individuals (Weng 2012).

An important factor influencing immune cell telomere length is disease itself. Observational studies on humans have found shorter immune cell telomere lengths in individuals suffering from age related diseases such as cardiovascular disease and cancer (Fossel 2012). A comparison of the immune cell telomere lengths of laboratory mice experimentally exposed to the infectious agent *Salmonella enterica* compared to uninfected controls has shown that telomere attrition can also occur as a direct consequence of infectious disease (Ilmonen *et al.* 2008). Despite the clear link between current disease status and immune cell telomere length, to date there have been no assessments of their relationship within a wild vertebrate population naturally infected with disease.

Here we use data from a longitudinal field study of wild European badgers (*Meles meles)* to investigate whether the within-individual age-related declines in immune cell telomere length observed in humans and laboratory models extend to a wild vertebrate population. European badgers are facultatively social and polygynandrous mammals that occupy underground dens (setts). Late life declines in reproductive success consistent with senescence have already been observed in European badgers (Dugdale *et al.* 2011b); however the physiological mechanisms contributing to such declines are currently unknown. As *Mycobacterium bovis* (the causative agent of bovine tuberculosis, bTB), is known to be present in our study population, we also investigate whether shorter immune cell telomeres are associated with disease in the wild.

Blood samples were collected from individually-marked badgers of known-age (ranging from 0.3 to 10.3 years) routinely trapped as part of a long-term study at Woodchester Park, Gloucestershire, UK (see Delahay *et al.* 2013 for methods). DNA was successfully extracted from 361 buffy coat samples collected from 173 badgers captured and sampled on 1-7 (median=2) separate occasions between May 2012 and October 2013. Longitudinal measures (individuals captured twice or more) were available for 88 individuals and represented 76% of the total number of observations in the dataset. Average immune cell relative telomere length was determined via a robust and repeatable relative qPCR approach then converted to an absolute telomere length measure (Kb) using standard methods (see Appendix A for complete methodology). The qPCR approach has been successfully utilised to estimate telomere length in numerous ecological studies (Heidinger *et al.* 2012; Barrett *et al.* 2012b; Nettle *et al.* 2013; Stier *et al.* 2014). It is particularly well suited to ecological field studies as it requires a small DNA sample, and is cheap and high-throughput in comparison to other methods available (Nussey *et al.* 2014). Furthermore, where results from qPCR assays have been compared to other methodologies (such as Terminal Restriction Fragment analysis) they are generally found to be well correlated e.g. (Aviv *et al.* 2011). That said, the qPCR technique cannot differentiate 'true' telomeric repeats from interstitial telomere-like repeats located away from the chromosome ends (Foote, Vleck & Vleck 2013). Whilst between-individual differences in the incidence of interstitial repeats have the potential therefore to add noise to comparisons of telomere length between groups of individuals, they are unlikely to influence the results of longitudinal studies (such as this one) where the within-individual change in telomere length is of principal importance (Nettle *et al.* 2013). It should also be noted that the absolute estimates of mean immune cell telomere length and telomere attrition rates presented should currently be treated with caution as they have not yet been verified with a second independent methodology (Horn, Robertson & Gemmell 2010; Nussey *et al.* 2014). That said, their inclusion here at least offers the potential for future comparisons with other studies employing a similar methodology (Barrett *et al.* 2012a). All work was approved by the Food and Environment Research Agency Ethical Review Committee and carried out

under licence granted by the Home Office under the 1986 Animal (Scientific Procedures) Act.

Infection Status

Current bTB infection status was assessed at each capture event using three diagnostic tests: i) the interferon-gamma (IFNγ) test, an enzyme immunoassay assessing lymphocyte responsiveness to an *M. bovis* purified protein derivative, ii) the STAT-PAK® (Chembio Diagnostic Systems, Inc) test, a lateral-flow immunoassay to identify the presence of *M. bovis* antibodies, and iii) microbiological culture of clinical samples (i.e. sputum, faeces, urine and wound/abscess swabs) to isolate *M. bovis* (see Chambers *et al.* 2002, 2009 for discussion of the performance of each test). We used a simplified version of the disease classification discussed in detail in Delahay *et al.* (2013). Briefly, individuals were classed as 'negative' if they had never tested positive for bTB on any test, 'positive' if they had ever tested positive using the STAT-PAK or IFNγ tests, or 'excretor' if *M. bovis* was isolated by culture. Thirteen individuals transitioned to a more advanced disease status during the course of the study: eleven individuals transitioned from disease 'negative' to 'positive', one individual from 'positive' to 'excretor' and one individual from 'negative' to 'excretor'. None of the individuals in this study had ever been vaccinated against bTB.

Seasonality-corrected body condition

In order to control for variation in body condition (which could conceivably be correlated with age or disease status) in the telomere length analysis (Caprioli *et al.* 2013), we calculated a metric for body condition that accounted for the marked seasonal variation in body mass exhibited by European badgers. To do so, we first calculated the Scaled Mass Index (SMI) for all individuals at each capture, following Peig & Green (2010). This approach factors out variation in body mass arising from variation in body size (in this case utilising body length (cm) as the metric of body size), by scaling the body mass measure for each capture to that for an individual of average size (in this case, of body length = 80cm). The SMI scaling factor was estimated to be 3.63. We then corrected all SMI measures for seasonal variation by taking residuals from a model of SMI that controlled for variation arising from the month of the year on which the individual was captured.

Statistical analyses

Competing hypotheses for the causes of variation in telomere length were compared using multi-model inference (Burnham & Anderson 2002) with linear mixed-models. *A priori* candidate models (n=28) were defined containing additive effects of all candidate explanatory variables: partitioned age (see explanation below), sex, bTB status, seasonally-corrected body condition and all biologically relevant two way interactions (see Appendix B for full table). A 'top model set' was defined, which included all models with ΔAICc≤6 from the best supported model, after excluding any models of which a simpler nested version attained stronger support (following the 'nesting rule' of Richards *et al.* 2011). All co-efficients were model-averaged across the models in the 'top model set' in which they occurred using the MuMIn package (Barton 2014). Model-averaged coefficients (effects sizes) are discussed in terms of their relative 'weights'. In order to ensure that our statistical assessment of the within-individual change in telomere length with age was not confounded by between-individual effects (e.g. selective disappearance), we applied a within-subject centring approach, following van de Pol & Wright (2009). Age was partitioned into (i) an individual's 'mean age' across all samples collected for that individual, and (ii) its 'Δ age' (the offset of its age at the focal sampling point from its mean age, the effect of which reflects within-individual changes in telomere length with age). Previous longitudinal and cross-sectional studies of immune cell telomere lengths have suggested that the rate of telomere attrition may slow with increasing age (Baerlocher *et al.* 2003; Aubert *et al.* 2012). To address this possibility, we assess the strength of evidence for quadratic effects of each partitioned age parameter and for an interaction between a mean age and Δ age. To account for repeated measures, qPCR plate heterogeneity, and variation in territory quality, we included 'individual ID' nested within 'plate ID' (all samples from the same individual were run on the same plate) and 'social group' as random intercept terms. Goodness-of-fit was assessed through calculating conditional (total variance explained by the best supported model) and marginal (variance explained by fixed effects alone) R^2 formulations (Nakagawa & Schielzeth 2013) and standard residual plot techniques.

The relationship between age and immune cell telomere length, after accounting for the effects of the random factors, is strongly suggestive of the predicted age-related decline (Figure 1A). Crucially, partitioning variance in age into within- and between-individual variation yielded full statistical support for age-related declines in telomere length occurring within individuals (Figure 1B and Table 1). The rate of telomere attrition with age occurring *within* individuals (mean±SE = 460±170 bp/year) was estimated to be over three times faster than that for *between*-individual increases in age (mean±SE = 140±40 bp/year; Table 1). We found no statistical support for quadratic age effects or an interaction between mean age and Δ age, suggesting that the rate of change in mean immune cell telomere length does not change with age.

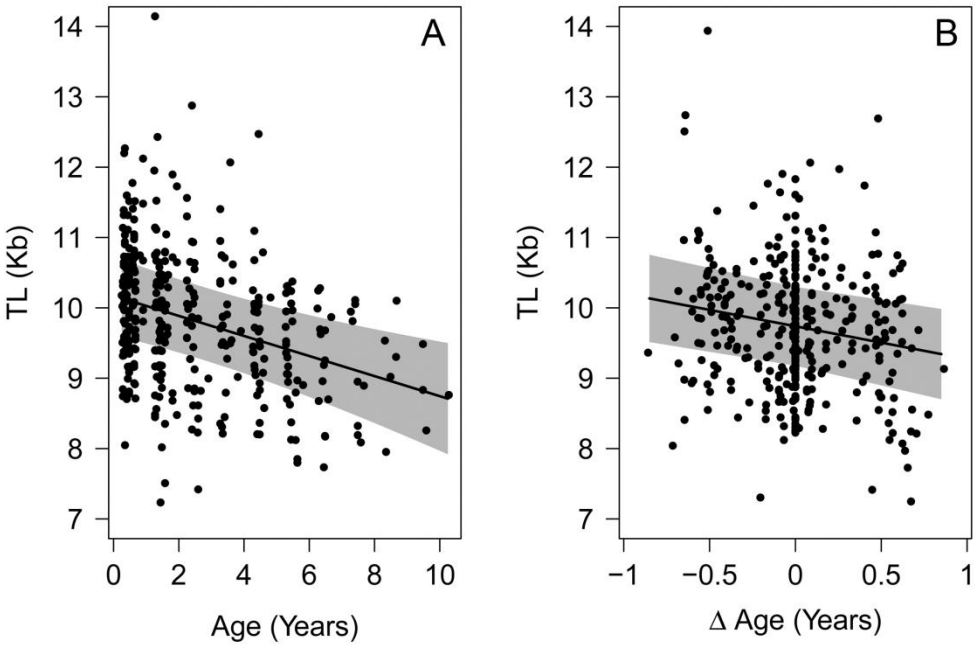


Figure 1: How telomere length changes with age and partitioned age. (A) Relationship between immune cell telomere length (TL) and age (years) whilst controlling for random effects; (B) Relationship between TL and Δ age (within-individual changes in age) while controlling for random effects, bTB infection status, and between-individual differences in age (Table 1); Black lines present model averaged predictions, shaded areas present 95% confidence intervals, and black points present residuals from the best supported models.

Table 1: Model averaged output of linear mixed model analysis of the factors affecting telomere length. Where; Σ = model weight; SE = effect size standard error; CI = confidence interval; [a]= factor levels. Interaction terms without support are not shown. For full model output see Appendix B.

Parameter	**Σ**	Effect size	SE	95% CI
Mean Age (Years)	1.0	-0.14	0.04	(-0.22 to -0.05)
Delta Age (Years)	1.0	-0.46	0.17	(-0.80 to -0.12)
Disease	0.7	-	-	-
[a]Negative		0.00	-	-
[a]Positive		0.24	0.23	(-0.03 to 0.74)
[a]Excretor		-0.26	0.37	(-1.13 to 0.40)
Sex	0.0	-	-	-
Condition	0.0	-	-	-

We also found some support for bTB infection status predicting telomere length (Table 1), whereby bTB positive individuals had longer telomere lengths than negative individuals, while individuals in advanced stages of infection ('excretors') showed shorter telomere lengths than badgers that tested positive (Figure 2). No support was found for bTB infection status influencing the rate of within-individual telomere attrition (an interaction between bTB status and Δ age). Likewise, no support was found for sex or current body condition influencing telomere length. The proportion of variance explained by the best performing model was 59.7%, though the fixed effects alone (age and disease) accounted for just 5.1% of the total variance.

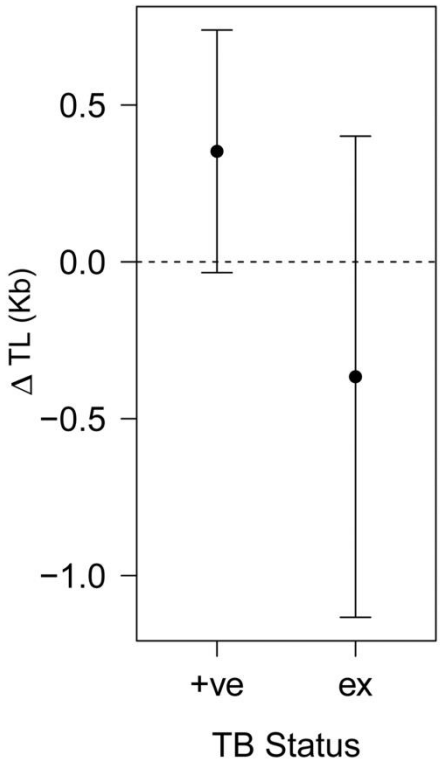


Figure 2: Predicted change in telomere length (TL) due to bTB infection status. Where the dashed line is the predicted TL of bTB negative individuals, '+ve' denotes disease positive individuals, and 'ex' individuals classed as excretors. The points present model averaged predictions and error bars present their 95% confidence intervals.

Our findings strongly suggest that the age-related declines in immune cell telomere length observed in humans and laboratory models are mirrored by comparable relationships in this population of wild mammals. These results therefore lend strength to the suggestion that age-related declines in immune cell telomere length may contribute to the emergence of the immunosenescent phenotypes increasingly documented in wild vertebrate populations (Palacios *et al.* 2011). Our findings are also suggestive of associations between immune cell telomere lengths and disease status in the wild, echoing the findings of biomedical research in which human immune cell telomere lengths have been identified as a risk factor for disease (Fossel 2012). Below we discuss the causal mechanisms that may underpin the relationships observed and their implications, in light of existing work on the telomere dynamics of other cell types in wild vertebrates, for our understanding of patterns of senescence in the wild.

Our findings suggest that the immune cell populations of wild European badgers experience a decline in mean telomere length of approximately 460bp/year with increasing organismal age. This rate of decline falls between recent estimates for the immune cell telomere attrition rates of humans (72bp/year) and laboratory mice (7000bp/year) (Vera *et al.* 2012) and is consistent with the previously documented negative correlation between telomere attrition rate and lifespan (Haussmann *et al.* 2003). The observed age-related declines in mean immune cell telomere length could principally reflect age-related changes in the telomere lengths of particular immune cell classes, the immunological implications of which will depend upon the cell class(es) involved. For example, the observed patterns may principally reflect age-related declines in the telomere lengths of neutrophils as they typically comprise around 80% of total immune cell counts in the European badger (McLaren *et al.* 2003; Tomlinson 2013). As the high turnover rate of neutrophils is thought to leave their telomere lengths reflecting those of the haematopoietic stem cells from which they derive (Goronzy *et al.* 2006; Aubert & Lansdorp 2008), it seems likely that our results reflect, at least in part, age-related declines in haematopoietic stem cell telomere length. To the extent that this is the case, our findings echo those of studies of wild birds, in which the

telomere lengths of nucleated erythrocytes (which also derive from the haematopoietic stem cells) have typically been found to decrease with age (Haussmann *et al.* 2003; Salomons *et al.* 2009; Barrett *et al.* 2012b). Collectively these findings highlight the possibility that pathological consequences of short telomeres in this stem cell compartment (Flores *et al.* 2006; Blasco 2007) may contribute to age-related declines in whole-organism performance. However, as lymphocytes also represent a significant proportion of European badger immune cells (~20%; McLaren *et al.* 2003; Tomlinson 2013), our findings may also reflect, at least in part, age-related declines in lymphocyte telomere lengths, whose dynamics may differ markedly from those of the haematopoietic stem cells. Age-related declines in the telomere lengths of lymphocytes *per se* have previously been documented in humans and laboratory models (Baerlocher *et al.* 2003; Alter *et al.* 2012), and, given the potential for short telomeres to limit lymphocyte proliferation capacity (Effros 2011), have been implicated as one potential driver of late-life declines in the capability of the adaptive immune system. The application of cell sorting to field-derived samples of immune cell populations prior to telomere analysis might now be usefully prioritised, to clarify which immune cell sub-types are exhibiting age-related declines in telomere length and, by extension, the immunological implications of such declines.

While our findings could indeed reflect age-related declines in the telomere lengths of specific immune cell subsets that might thereby contribute to the emergence of immunosenescent phenotypes, there are several reasons for caution when considering this possibility. First, it is also possible that the observed age-related changes in immune cell mean telomere length instead arise in part from changes with age in the ratios of immune cell sub-types in circulation (Nussey *et al.* 2012), which may differ in mean telomere length (e.g. an increasing representation of memory T-cells with shorter telomeres relative to naïve T-cells with longer telomeres; Weng 2012). Ultimately, the relative contributions of telomere attrition *per se* versus age-related changes in immune cell composition will only become clear once cell-type-specific telomere length estimates can be coupled with knowledge of age-related changes in the ratios of these same cell types, which is currently an ambitious prospect for studies of non-model organisms. Second, even if the observed age-related changes do arise from within-cell-type changes in telomere length, our statistical models highlight that the fixed effects alone (age and disease status) explain only

a small proportion of the observed variation in mean immune cell telomere length (just 5%), suggesting that the biological significance of these within-individual changes with age may be limited relative, for example, to the processes that generate between-individual variation. Finally, while the observed age-related declines in mean telomere length could be a pathological consequence of cumulative exposure to the diverse processes that may shorten immune cell telomere lengths (e.g. oxidative stress or disease), it is also possible that they reflect, in part or whole, adaptive changes in immunological investment with age (either in telomere maintenance or immune cell sub-type ratios), given, for example, a reduced likelihood that older individuals will encounter novel pathogens. Whether naturally occurring age-related changes in immune cell telomere length necessarily would contribute to late-life declines in whole organism performance in natural populations under ecologically realistic conditions therefore remains open to debate.

Our findings are also suggestive of an association between disease state and immune cell telomere length in a natural vertebrate population under ecologically realistic conditions, again echoing studies of humans and laboratory models (Ilmonen *et al.* 2008) However, it must be noted that due to the low frequency of indviduals transitiong between disease states within the study period these patterns are largely cross-sectional in nature. The initial increase in telomere length with the transition to testing positive for bTB was unexpected; however, previous work in humans found telomerase activity to be present in immune cells of individuals with pulmonary tuberculosis and absent in healthy controls (Yang *et al.* 1999). As the immune responses to bTB comprise of both cell-mediated and humoral components (Flynn & Chan 2001), involving T and B-cells which are known to express telomerase (Weng, Granger & Hodes 1997; Weng 2012), telomerase expression in immune cell subsets during infection may be contributing to this pattern. The apparent decrease in immune cell telomere length associated with progression from testing positive for bTB to excreting the bacterium is consistent with evidence that disease progression in humans is linked to the shortening of immune cell telomeres (Armanios 2013). Whether this association reflects short telomeres pre-disposing individuals to disease progression (Weng 2012), the impacts of infection on immune cell telomere length (Ilmonen *et al.* 2008), or indeed a role for other processes that may both shorten immune cell telomeres and leave individuals pre-disposed to disease progression, remains unclear. It is also conceivable that these patterns reflect

disease-associated variation in the composition of the immune cell population from which our telomere length assessments derive, rather than variation in the telomere lengths of specific cell types. As the presence of critically short telomeres (rather than an overall decline in population average telomere length) may act as the crucial factor linking disease progression and immune cell viability (Hemann *et al.* 2001; Lin *et al.* 2014), this possible link between disease status and telomere dynamics may be more appropriately addressed using methodologies with single chromosome resolution (Nussey *et al.* 2014).

Our findings have implications too for a growing body of work seeking to understand the causes and consequences of sex differences in telomere dynamics (Barrett & Richardson 2011). It has been argued, for example, that males might be expected to show shorter telomeres and/or experience higher rates of telomere attrition in species that exhibit male-biased sexual size dimorphism, male-biased mortality rates and/or steeper rates of senescence decline among males than females (see Barrett & Richardson for a review). As all three phenomena have been documented in the European badger (Johnson & Macdonald 2001; Dugdale *et al.* 2011b; Graham *et al.* 2013), it is notable that our findings reveal no evidence of a sex difference in either mean immune cell telomere length or in the within-individual rate of immune cell telomere attrition with age. Our findings therefore echo recent work highlighting the likely complexity of the proximate and ultimate causes of sex differences in telomere dynamics, and the difficulty of drawing generalisations from the small number of studies in this area to date (Barrett & Richardson 2011).

In summary, our findings provide the first evidence, to our knowledge, of within-individual declines in immune cell telomere length with age in a wild vertebrate, suggesting that the immune cell telomere attrition documented in humans and laboratory models may indeed generalise to natural vertebrate populations experiencing ecologically realistic conditions. As such, while their immunological implications will only become clear with further research, our findings do lend strength to the view that age-related declines in immune cell telomere length may offer one potentially general mechanism contributing to declines in both immune and whole-organism performance with age. It remains to be tested whether short immune cell telomeres are associated with weaker whole-organism performance in the wild as appears to be the case for erythrocyte telomere length in wild birds (Geiger *et al.*

2011; Barrett *et al.* 2012b; Angelier *et al.* 2013). However, that our statistical models estimate the rate of within-individual age-related decline in immune cell telomere length to be roughly three times faster than the rate of between-individual decline is at least consistent with a scenario where individuals with shorter immune cell telomeres and/or faster telomere attrition rates are selectively disappearing from this population. A key challenge now will be establishing the causes of variation among individuals in the rate of immune cell telomere attrition with age, and the extent to which such variation has causal effects on late-life health and performance.

Chapter 3: Early life environmental conditions predict adult telomere lengths in wild European badgers

Abstract

The conditions experienced in early-life are now known to contribute to the marked inter-individual variation in senescence rates observed in nature; however the mechanisms underpinning such effects remain poorly understood. One way in which natural variation in early life conditions is thought to affect rates of late-life deterioration is through influencing telomere dynamics. Here we examine how natural variation in environmental quality, population density and growth rate in early life influence adult immune cell telomere length in the European badger (*Meles meles*). We show that individuals that experienced favourable environmental conditions (higher August rainfall) in their first year of life had longer telomeres in adulthood than those that experienced poor conditions in early life. The role of early-life environmental conditions was independent of local population density and individual growth rates, each of which had no detectable effect on adult telomere dynamics. To our knowledge, this is the first evidence of a link between early-life conditions and adult telomere length in a wild mammal. Our research lends strength to the view that telomere dynamics may provide a mechanistic link between early experiences and late life declines in performance in the wild.

Senescence, the within-individual deterioration in performance with increasing chronological age, has been documented in a wide range of free-living vertebrates (Nussey *et al.* 2008, 2013). Where explored, senescence has been found to be a plastic trait, often exhibiting considerable intra-specific variation in its age of onset and rate of progression (Nussey *et al.* 2013, Chapter 5). Life history theories of ageing suggest that variation in senescence rates arise from resource allocation trade-offs occurring between somatic maintenance and other competing functions (e.g. early life growth and reproduction; Williams 1957; Kirkwood 1977). All other things being equal, an individual that increases allocation of resources to early life growth or reproduction will have a reduced budget for somatic maintenance over the same period (Kirkwood & Rose 1991). Reduced investment in somatic maintenance will result in the accumulation of unrepaired cellular damage which may pre-dispose individuals to earlier onset, or more rapid progression, of senescent declines in late life (Monaghan *et al.* 2008). Thus, individual variation in senescence rates should arise with variation in any factor which influences an individual's investment in somatic maintenance. Determining the physiological mechanisms that underpin the links between early life investment in somatic maintenance and late-life declines in performance is a key focus for researchers in evolutionary ecology looking to understand senescence and its implications for host fitness (Monaghan *et al.* 2008).

One factor implicated in influencing senescence rates, particularly in wild populations, is environmental stress experienced during growth and development (e.g. food availability and climatic conditions; Nussey *et al.* 2007). Individuals experiencing 'poor' conditions where resources are scarce (e.g. due to high population density or low food availability) or the climate is harsh (e.g. leading to high resource demands) will have access to a smaller resource pool available for allocation to somatic maintenance and other key functions, such as growth and immunity. As such, investment in functions other than somatic maintenance during development will come at the detriment of cell repair, leading to delayed effects of early life environmental conditions on late-life performance (Kirkwood 1977). Consistent with this, individuals that experienced harsher early life environmental conditions in the

wild have been found to show faster rates of senescence, such factors include high population density (e.g. Nussey *et al.* 2007) and harsh weather conditions (e.g. Reed *et al.* 2008).

In addition to the environmental conditions experienced, investment in early life growth can also trade-off with early life somatic maintenance, and thus has implications for late-life declines in individual performance (Metcalfe & Monaghan 2003). Whilst one might assume that organisms should grow as quickly as possible, given the potential benefits of doing so (e.g. improved survival and competitive ability), they rarely grow at their maximum rate in the wild (Metcalfe & Monaghan 2003). This is due to both short-term (e.g. increased exposure to predators; Arendt 1997) and long term costs of doing so (e.g. accumulation of cellular damage due to oxidative stress; Kim *et al.* 2010). Support for the hypothesis that rapid growth trades off against long term somatic maintenance and longevity has been found in both laboratory settings (e.g. Rollo 2002; Ozanne & Hales 2004; Lee *et al.* 2013) and in the wild (e.g. Geiger *et al.* 2011). However, these studies focus on the special case of 'catch-up growth', where individuals grow rapidly to compensate for a period of growth delay due marked nutritional or environmental stress. Whether such growth/somatic maintenance trade-offs are manifest in the normal among-individual variation of growth rates remains an open question. Growth/somatic maintenance trade-offs may not be apparent when examining natural among-individual variation in growth rates as individuals may vary, for example, in their overall access to resources and simply grow as fast as they can while limiting costs to somatic maintenance (Metcalfe & Monaghan 2003). It could also be the case that the effects of rapid growth only become apparent if stressful environmental conditions are experienced in early life (Mangel 2008). Stressful conditions may act to exacerbate growth/somatic maintenance trade-offs if, for example, they reduce the overall size of resource pool available for allocation at that time (Mangel 2008).

One candidate mechanism linking early life growth and environmental conditions with late-life declines in performance is telomere length decline (Monaghan 2010). Telomeres are protective nucleoprotein complexes comprising of $(TTAGGG)^n$ repeat sequences found at the end of all vertebrate chromosomes. At the cellular level they are crucial for genome stability and the maintenance of replicative capacity (Chan & Blackburn 2004). At the whole

organism level average telomere length has been found to predict survival and longevity (Salomons *et al.* 2009; Heidinger *et al.* 2012; Barrett *et al.* 2012b). Rapid early life growth rates or stress resulting from harsh environmental conditions could lead to increased production of reactive oxygen species (Nussey *et al.* 2009b; Kim *et al.* 2010) which have been linked with telomere erosion both *in vitro* (von Zglinicki 2002) and *in vivo* (Geiger *et al.* 2011). Telomere erosion also occurs with repeated cell divisions (owing to the end replication problem; Shay & Wright 2000) and thus individuals that reach larger overall body sizes may have shorter adult telomeres than smaller individuals regardless of the oxidative burden experienced or the rate grown to reach that size (Stindl 2004; Barrett & Richardson 2011). Rapid early life growth has been shown to increase oxidative stress levels, reduce telomere length and reduce life span in laboratory rats (Jennings *et al.* 1999; Tarry-Adkins *et al.* 2009). However, just a handful of studies have examined the effects of early life environmental conditions and growth on telomere dynamics in the wild, all of which have been conducted on birds (Hall *et al.* 2004; Foote *et al.* 2011; Geiger *et al.* 2011; Voillemot *et al.* 2012). To date, no studies have investigated whether early life conditions and growth rates predict adult telomere lengths in wild mammals.

Here we examine the effects of natural variation in early life environmental conditions and growth trajectories on the adult immune cell telomere length and attrition rate of European Badgers, *Meles meles.* Both reproductive senescence (Dugdale *et al.* 2011b) and marked inter-individual variation in body mass senescence rates (Chapter 5) are known to occur within the European badger. Furthermore, substantial inter-individual variation in adult immune cell telomere length has been documented within this population (Chapter 2), but the mechanisms driving such variation remain unknown. The environmental conditions experienced in early life represent a likely candidate for influencing adult telomere lengths in the European badger as they are known to be important determinants of cub survival and recruitment (Woodroffe & Macdonald 2000; Macdonald *et al.* 2010). Given that both sex-specific reproductive senescence (Dugdale *et al.* 2011b) and body mass-senescence (Chapter 5) have been documented, and that European Badgers show sexual size dimorphism (males are typically larger than females; Roper 2010) we also assess the evidence for sex-specific trade-offs between telomere length and early life conditions.

Specifically we determine: 1) if poor environmental conditions experienced in early life predict adult telomere length, 2) if individuals that grow faster or attain larger adult sizes show reduced immune cell telomere lengths in adulthood 3), if there are sex-specific costs to environmental conditions, rapid growth or attaining a larger adult size. Two environmental metrics (August rainfall and May rainfall) were used as proxies for the quality of early life physical environment, based on previous findings that (i) higher August rainfall is associated with higher cub and adult survival (through its positive effect on food availability; Woodroffe & Macdonald 2000), and (ii) higher May rainfall is associated with *poorer* cub survival (conceivably due to costs of young cubs being soaked in the cooler part of the year; Macdonald *et al.* 2010). We explored the evidence for density dependent costs of environment quality, by investigating whether an interaction between rainfall patterns and local badger density predicts adult telomere length. Finally, we assessed the evidence for environment-dependent costs of high growth rates, by investigating whether interactions between the early life environment and growth rate predict adult telomere length.

Study Site and Population

All research was conducted on the high-density population of European badgers (*Meles meles*) at Woodchester Park, Gloucestershire, UK. The resident population has been the subject of continuous epidemiological monitoring since the mid 1970s. During the spring of each year, the boundaries of each badger social group territory were approximated by bait marking and traps deployed at each active sett for two nights, four times per year (Delahay *et al.* 2000). All badgers captured between January 2002 and October 2013 were anaesthetised and identified through a unique tattoo code administered at their first capture. The date, location, sex, length (to the nearest cm – from the tip of the nose to the distal point of the last caudal vertebra), mass (to the nearest 100g) and age class (juveniles < 1 year, adult ≥ 1) were recorded for each individual. Captures of individuals of unknown age (those that were not identified as juveniles at their first capture) or social group were excluded from the analysis. For individuals that were first captured as juveniles, age is defined as the number of days elapsed since 20th February in their first year of life, which reflects the mid-February peak in births (exact dates of birth cannot be readily determined; Roper 2010). At each capture, biological samples were taken from each individual for the determination of bovine tuberculosis (bTB) infection status using a suite of tests (Delahay *et al.* 2013). Briefly, individuals were classed as bTB 'negative' if they had never tested positive for bTB on any test, 'positive' if their blood had ever tested positive using the STAT-PAK or IFNγ tests (Chambers *et al.* 2009), or 'excretor' if *Mycobacterium bovis* had ever been isolated by culture from blood, sputum, faeces, urine or wound swabs (Chambers *et al.* 2009). We used a one-way progressive system where by individuals can transition to elevated disease classifications when appropriate tests are positive, but can never return to lower classifications (as in Tomlinson *et al.* 2013).

Telomere length

Between January 2011 and October 2013, DNA was successfully extracted from the blood samples of 173 unique individuals of known age that were captured on one or more occasions (totalling 361 capture events). Extracted DNA was stored at -20°C until they were

assayed for absolute immune cell telomere lengths using a robust and repeatable absolute qPCR technique (see Chapter 2; Appendix A). The telomere length data set utilised here represents a subset of the data used in Chapter 2, comprising only of telomere measurements taken from adults (>365 days old) for whom early life growth rates could be calculated (i.e. they were captured on more than one occasion in the growth season of their first year of life; see below for details). This dataset comprises 177 adult telomere length measurements from 76 unique individuals, comprising of 42 females and 34 males (median observations per individual = 2; range = 1-7).

Environmental Conditions

We used May and August rainfall in the first year of life as proxies for the quality of the environmental conditions experienced in early life, as each has been previously linked to cub survival (Woodroffe & Macdonald 2000; Macdonald *et al.* 2010). Higher August rainfall has been found to positively predict the proportion of cubs surviving to adulthood; a relationship that is thought to be mediated through a positive effect of rainfall at this time on resource availability (Woodroffe & Macdonald 2000). By contrast, evidence that May rainfall *negatively* predicts the number of cubs recruited into the population has been hypothesised to reflect a negative effect of May rainfall on cub survival, mediated by costs arising from cubs being soaked during the crucial weaning period (Macdonald *et al.* 2010). As there is no permanent weather station based at the Woodchester Park field site, May rainfall and August rainfall data were obtained from the nearest available weather stations: Avening (2002-2007) and Eastington (2008-2013); both of which are within a 5 mile radius of the study site.

Local population density

Local population density in each individual's first year of life was estimated by first calculating an annual value for the local density of all individuals around each badger sett, defined as the total number of unique badgers caught within a 280m radius of each sett in each year. This search radius provides coverage equal to the mean territory size of a social group across the study period (24.5 hectares), and thus constitutes a likely spatial scale over which individuals will interact. Local density was used, rather than social group size (the

number of unique individuals caught within a social group territory in each year), as between group contacts are common (Dugdale *et al.* 2007) and local density makes fewer assumptions about the relative contribution of intra- or extra-group competition for resources. When a badger was caught at only one sett during its first year of life, the local population density represents the number of unique badgers caught within 280m of that sett in that year. When a badger was caught at multiple setts during its first year of life, a weighted average population density was calculated based on the number of times that it was caught at each sett.

Estimation of early life growth rate and final adult size

For individuals that were caught on more than one occasion during their first year of life it was possible to determine their body length growth rate. Age was $\log_{10}$ transformed in order to account for growth slowing towards the end of the growth period (see Appendix C). Growth rate was defined as the gradient of the relationship between body length (cm) and $\log_{10}$(age) as determined by a linear regression for all individuals with more than one capture between 76 and 300 days of age (see appendix D). While more complex equations are available for the analysis of growth (e.g. López *et al.* 2000; English *et al.* 2012), they typically require substantially more frequent measures in order to successfully parameterise. No captures were made before 76 days of age due to welfare restrictions on trapping cubs still dependent on lactation. Captures after 300 days of age were excluded as they violated the log-linear relationship between age and length and thus reliable estimates of growth rate could not be obtained (see Appendix C). This allowed us to calculate growth rates for 80 individuals, each of whom had 2 or more body length measures available between 75 and 300 days of age (median = 2, Range = 2 to 4). Four of these individuals were excluded from the analysis as they were found to show negligible or negative growth during the focal growth period (see appendix D) indicating that they had already completed their growth by the time of sampling, been misclassified as juveniles or had their length recorded inaccurately. We used body length for the calculation of growth rates, rather than body mass, as it reflects irreversible 'structural growth' and avoids the problems that would arise from the marked seasonal variation in body mass exhibited by this species. All analyses were, however, repeated using growth rates calculated using body mass (kg) data in place of body length (cm). As this approach yielded qualitatively similar statistical findings to those

obtained using body length, only the findings arising from the body length analyses are presented. For analyses of the effect of final adult size on adult telomere length, final adult size was defined as the average length from all the captures over the focal individual's adult lifetime (mean = 84.0cm; average Co-efficient of Variation (CV) = 0.009).

Statistical Analysis

Prior to determining the factors which influence adult immune cell telomere length, we first determined if the trait showed repeatable between-individual variation. We calculated raw phenotypic variability following Nakagawa & Schielzeth (2010) using a linear mixed-effect model with immune cell telomere length as the response variable and 'individual ID' nested within 'plate ID' (as all of the samples from each individual were run on the same qPCR plate) and 'social group' as random intercept terms. Variance components were estimated using restricted maximum likelihood. 95% confidence intervals for the repeatability estimate were generated using parametric bootstrapping (Nakagawa & Schielzeth 2010).

In order to examine the early life drivers of adult telomere length we implemented a multi-model inference approach (Burnham & Anderson 2002) using linear mixed-models. Our primary predictors of interest (the early-life rainfall measures, population density, growth rate and adult size) were all standardised and scaled to unit variance. We also examined the explanatory power of the intercept of the growth regression line for each individual, "growth intercept" (again standardised and scaled to unit variance), to allow for the possibility of an additive effect of an individual's initial size prior to our growth rate calculation on their adult telomere length (though no such effects were detected). Average immune cell telomere length was the response term in all models. As we have previously shown that immune cell telomere length in this species declines with age and varies according to current bTB status (Chapter 2), age and current bTB status were included as predictors in all models. Age was partitioned into (i) an individual's 'mean age' across all samples from that individual that featured in the analysis, and (ii) 'Δ age' (the offset of the individual's age at the focal sampling point from its mean age, the effect of which reflects within-individual changes in telomere length with age) following van de Pol & Wright (2009). This approach allows us to examine the effects of any predictors on the rate of within-individual change in telomere length with age, by fitting interactions between the focal

predictor and Δ age. A series of *a priori* candidate models (n=44) were defined containing additive effects of all candidate covariates and all biologically relevant two-way interactions (see Appendix S3 for full model specifications). Models were ranked and a 'top model set' was defined, which included all models whose AICc scores fell within 6 units of the best supported model, after excluding any models of which a simpler nested version attained stronger support (following the 'nesting rule' of Richards *et al.* 2011). Effect size coefficients were model-averaged across all of the 'top set' models in which they occur using the MuMIn package (Barton 2014). From the ΔAICc score of each model in the 'top set', an Akaike weight was calculated which represents the probability that a given model is the best, relative to all other models in the 'top set' (Burnham, Anderson & Huyvaert 2010). Covariates were then ranked by calculating the sum of the Akaike weights for each model in the 'top set' in which it occurs, and are discussed in terms of this relative 'weight'. The larger the relative weight of the covariate, the stronger the evidence for its effect relative to the other covariates. To account for repeated measures of individuals, heterogeneity between telomere qPCR assay plates, and variation in territory quality, we included 'individual ID' nested within 'plate ID' (as all of the samples from each individual were run on the same qPCR plate) and 'social group' as random intercept terms in all models. Goodness-of-fit was assessed by calculating conditional (total variance explained by the best supported model) and marginal (variance explained by fixed effects alone) R^2 formulations (Nakagawa & Schielzeth 2013) and standard residual plot techniques.

We first confirmed that adult immune cell telomere length showed repeatable between-individual differences (repeatability = 0.403, 95% Confidence Interval = 0.14-0.56). While controlling for the known effects of age and bTB infection status on immune cell telomere length, we found that early life environmental conditions predicted immune cell telomere lengths in adulthood. Individuals that experienced higher August rainfall in their first year of life (which has been shown to positively predict cub survival; Woodroffe & Macdonald 2000) had longer immune cell telomere lengths in adulthood (Figure 1A; Table 1). We found no evidence of an effect of either early life May rainfall (which has been hypothesised to influence cub survival; Macdonald *et al.* 2010) or early life local population density on adult telomere length (Figure 1B and 1C; Table 1). We also found no evidence of sex-specific effects of the early life environment or that the early life environment influenced the rate of within-individual telomere attrition rate as an adult (i.e. interactions between the early life predictors and both sex and Δ age were uninformative; Table 1). The best supported model explained 60.3% of the model variance (conditional R^2), and the fixed effects alone accounted for 11.1% of the total variance (marginal R^2).

We found no statistical support for the hypotheses that individuals that grew more rapidly or were larger as adults had shorter immune cell telomere lengths in adulthood than those that grew slowly or were smaller as adults (Figure 2A and 2B). There was also no evidence for environment dependent costs of rapid growth (i.e. individuals growing rapidly in poor quality years showed no detectable loss of adult telomere length; Table 1). Furthermore, we found no evidence for sex-specific impacts on telomere lengths in adulthood of either rapid growth or the attainment of larger adult size (interactions between sex and both growth rate and adult body size were uninformative; Table 1). Finally we found no evidence to suggest that the within-individual telomere attrition rate in adulthood was faster within individuals who had grown at faster rates or who had reached larger adult sizes (interactions between Δ age and both growth rate and adult body size were uninformative; Table 1).

Table 1: Model averaged output of linear mixed model analysis of the early life growth parameters affecting adult telomere length. Where: Σ = relative weight, β-Estimate = model averaged effects size, SE = effect size standard error, CI = confidence interval, a= factor levels. The grey area denotes parameters involved in model selection. Bold typeface denotes parameters with support (included in the top model set). Parameters including the "growth intercept" term are not shown. For full model output see Appendix E.

Parameter	**Σ**	B-Estimate	SE	95% CI
Δ Age	1.00	-0.49	0.23	-0.940 to -0.041
Mean Age	1.00	-0.12	0.06	-0.242 to 0.011
bTB Status (Negative)	1.00	-	-	-
- Positive	-	0.41	0.32	-0.217 to 1.031
- Excretor	-	-1.07	0.57	-2.195 to 0.052
August Rain	0.89	0.38	0.14	0.105 to 0.646
May Rain	0.00	-	-	-
Adult Size	0.00	-	-	-
Growth Rate	0.00	-	-	-
Density	0.00	-	-	-
May Rain * Adult Size	0.00	-	-	-
August Rain * Adult Size	0.00	-	-	-
May Rain * Growth Rate	0.00	-	-	-
August Rain * Growth Rate	0.00	-	-	-
May Rain * Sex	0.00	-	-	-
August Rain * Sex	0.00	-	-	-
May Rain * Δ Age	0.00	-	-	-
August Rain * Δ Age	0.00	-	-	-
May Temp * bTB Status	0.00	-	-	-
August Rain * bTB Status	0.00	-	-	-
May Temp * Density	0.00	-	-	-
August Rain * Density	0.00	-	-	-
Growth Rate * Δ Age	0.00	-	-	-
Growth Rate * Sex	0.00	-	-	-
Growth Rate * bTB Status	0.00	-	-	-
Growth Rate * Adult Length	0.00	-	-	-
Density * Δ Age	0.00	-	-	-
Density * Sex	0.00	-	-	-
Density * bTB Status	0.00	-	-	-
Density * Adult Length	0.00	-	-	-

Figure 1: Model averaged predictions for the environmental factors hypothesised to influence adult telomere length. (A) presents the predicted adult telomere length for individuals experiencing different environmental conditions (total August rainfall) in early life. (B), (C) present the lack of relationship between adult telomere length and May rainfall (B) and local badger density (C). The points denote residuals from the best supported model, the grey polygon denotes 95% confidence intervals, the black lines denotes the model averaged predictions for a disease negative individual at 3.5 years of age, the dashed lines denote the mean predicted telomere length for an individual at 3.5 years of age.

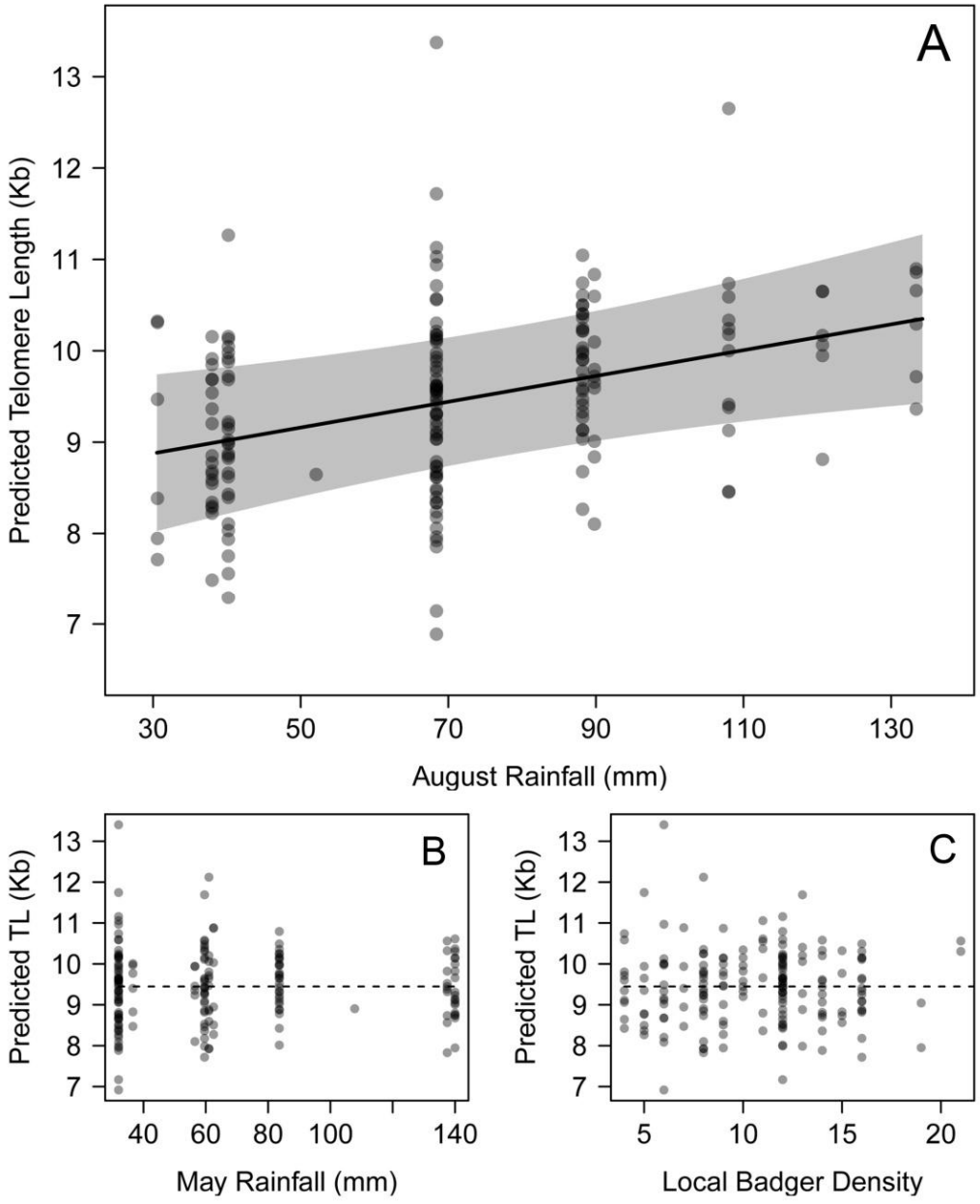
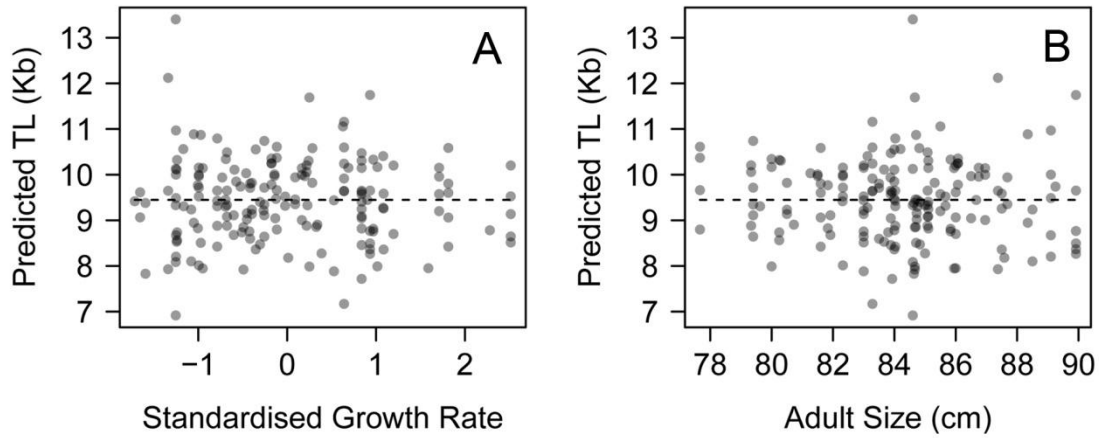


Figure 2: No relationship between growth factors and adult telomere length. (A), (B) present the lack of relationship between adult telomere length and standardised growth rate (A) and local badger density (B). The points denote residuals from the best supported model, the dashed lines denote the mean predicted telomere length for an individual at 3.5 years of age.



Whilst the conditions that individuals experience in early life have been implicated in influencing their senescence trajectories (e.g. Nussey *et al.* 2007), the mechanisms underpinning such links are poorly understood (Monaghan 2008). One candidate mechanism linking early life experience with senescent decline in late life is telomere attrition (Monaghan & Haussmann 2006). Here we investigated how early life conditions and individual growth rates influence adult immune cell telomere length in a wild population of European badgers (*Meles meles*). Whilst controlling for the previously documented effects of age and disease (Chapter 2), our analyses strongly suggest that variation in adult telomere length can arise from the conditions that individuals experienced in their first year of life. Individuals that experienced higher August rainfall as juveniles (which has previously been shown to positively predict cub survival; Woodroffe & Macdonald 2000) had longer immune cell telomere lengths in adulthood. This finding, the first of its kind for wild mammals, supports the current evidence available from both wild (Hall *et al.* 2004; Foote *et al.* 2011; Geiger *et al.* 2011; Nettle *et al.* 2013; Boonekamp *et al.* 2014) and domestic birds (e.g. Haussmann *et al.* 2012) in suggesting long-term impacts of early life conditions on telomere length. Investment in early life growth also trade-offs with early life somatic maintenance and thus has implications for late-life declines in individual performance (Metcalfe & Monaghan 2003). However, we find no evidence to suggest that natural variation in growth rate or adult body size influenced adult telomere length in this population. Below we discuss the causal mechanisms that may underpin the relationships observed and their implications for our understanding of senescence in the wild.

Our findings that individuals who experienced favourable environmental conditions (higher August rainfall) in their first year of life had longer immune cell telomeres in adulthood is consistent with a growing body of evidence suggesting that the early-life environment is a crucial determinant of future life history trajectories (Wilkin & Sheldon 2009; Lee *et al.* 2013). While our findings are the first of their kind for wild mammals, they parallel recent evidence in the biomedical literature linking stressful childhood environments with reduced adult immune cell telomere lengths in humans (e.g. Kiecolt-Glaser *et al.* 2011; Tyrka *et al.*

2011) and stress and telomere attrition in captive mice (Kotrschal *et al.* 2007). We used august rainfall as a proxy for inter-annual variation in resource availability, as it has previously been found to positively predict both cub and adult survival in another high density badger population in the UK (Woodroffe & Macdonald 2000). The reduced adult telomere lengths associated with low August rainfall may therefore reflect impacts of nutritional stress and/or social stress arising from foraging competition on the patterns of somatic maintenance in early life. The impact of early life stress could conceivably be mediated by elevated circulating concentrations of glucocorticoid stress hormones (Haussmann & Marchetto 2010). Glucocorticoids have diverse physiological effects that facilitate coping with adversity, but elevated glucocorticoid concentrations can cause telomere degradation, either by promoting the production of reactive oxygen species and so increasing the risk of oxidative stress, or by down-regulating the expression and/or efficacy of telomerase, an enzyme which can lengthen telomeres *in vivo* (Choi, Fauce & Effros 2008; Haussmann & Marchetto 2010). It is also conceivable, however, that harsh early life conditions could influence adult telomere lengths not through immediate effects on early life somatic maintenance, but through downstream effects on aspects of early adulthood life-history (e.g. on the costs and timing of first reproduction; Reed *et al.* 2008; Cartwright *et al.* 2014), that might themselves impact patterns of somatic maintenance in adulthood. Regardless of the mechanism by which they arise, reduced immune cell telomere lengths in adulthood are likely to have detrimental effects in late-life. Reduced immune cell telomere length is a predictor of age-related disease, mortality and immunosenescence in humans (Effros 2011; Fossel 2012) and reduced erythrocyte telomere length has been linked to mortality and longevity in birds (e.g. Barrett *et al.* 2012; Heidinger *et al.* 2012). It remains to be demonstrated whether individuals with shorter average immune cell telomere lengths in this population have reduced survival probabilities, increased disease susceptibilities or accelerated rates of senescence. However, that the within-individual rate of change in telomere length with age is faster than that at the population level (Chapter 2) is at least consistent with the selective disappearance from this population of individuals with shorter telomeres.

Whilst we found a strong effect of early life August rainfall on adult immune cell telomere length, we found no effect of early life May rainfall or local population density. The link

between May rainfall and cub survival was inferred from recruitment into the population being lower when May rainfall was high (Macdonald *et al.* 2010). As such, it may not be as strong a predictor of early life stress. High May rainfall could be correlated with the pre-natal environment (e.g. on litter size or viability), rather than post-natal effects on cub survival *per se*, as cub survival was never directly examined. Despite the previously documented links between early life population density and senescence rates in other species (e.g. Nussey *et al.* 2007), we found no evidence of a downstream effect of early life local density on the adult telomere length of individuals in this population. There are also other notable examples in the wild vertebrate literature of a lack of relationship between natal environment and senescence rates: Descamps *et al.* (2008) found evidence for survival and reproductive senescence in the red squirrels, but no evidence that food availability in early life influenced senescence rates and Millon *et al.* (2011) found no impact of prey density at birth on the senescence rates of tawny owls.

Previous research in controlled conditions (Jennings *et al.* 1999; Tarry-Adkins *et al.* 2009) and the wild (Geiger *et al.* 2011) collectively support the idea that investment in accelerated growth can trade-off against investment in somatic maintenance. Ours is the first study to investigate whether such a trade-off manifests in a wild mammal as a negative correlation between natural variation in growth rates and telomere lengths in adulthood, and we found no evidence that this was the case. Neither the rate at which individuals grew or the size that they ultimately attained predicted their immune cell telomere lengths in adulthood or the rate of within-individual telomere length decline. The disparity between our findings and those previously detailed may be due to the majority of studies focusing on the phenomena of "catch up" growth after a period of experimental or natural growth restriction (e.g. Tarry-Adkins *et al.* 2009; Geiger *et al.* 2011), rather than natural variation in unrestricted growth rates. In this population, while individuals with 'small' growth intercepts (which may be expected to scale with an individual's initial size) do appear to show faster rates of growth, they reach, on average, smaller adult sizes than their 'larger' counterparts (see Appendix F). Thus small individuals investing in rapid growth may only do so if costs to somatic maintenance are minimal. The lack of effect of growth parameters could also be due to individual quality differences whereby better quality individuals may, for example, have access to more resources and thus have more to allocate to both growth

and telomere maintenance (Metcalfe & Monaghan 2003). It is also the case that immune cells (the cell population used here for the calculation of telomere lengths) comprise of a diverse composition of cells, some of which are known to express telomerase (Nussey *et al.* 2014). Telomerase expression could preclude the detection of telomere attrition occurring as a direct cost to growth if, for example, rapid growth was associated with increased telomerase activity. This may explain the disparity between this work and research conducted on birds (as avian erythrocytes are not known to express telomerase; Nussey *et al.* 2014). It is also possible that immune cells may not be as an appropriate tissue to study the costs of rapid growth, as for example, directly measuring the telomere length of the stem cells involved in structural tissue production. Whatever the cause, the lack of covariance between growth parameters and adult telomere length in this population of mammals suggests that any positive fitness consequences arising from rapid growth or attaining a larger size (e.g. for reproductive success) do not appear to be accompanied by negative fitness consequences that might arise from reduced telomere lengths (e.g. reduced survival / lifespan).

European badgers display sex-specific senescence rates (males senesce faster than females; Dugdale *et al.* 2011; Chapter 5), sexual size dimorphism (males are typically larger than females; Roper 2010) and sex-specific adult mortality (survival is lower in males than females; Graham *et al.* 2013). It has been previously hypothesised that sex-specific growth trajectories or sensitivity to early life conditions could contribute to why sex differences in adult telomere length, although not ubiquitous, are often found in nature (Barrett & Richardson 2011). We find no evidence for sex differences in adult telomere length (Chapter 2), telomere attrition rate (Chapter 2) or in the effects of early life environment on telomere length. Taken together, this suggests that the sex differences in senescence rates and adult mortality in the European badger are not a product of sex differences in *immune cell* telomere dynamics *per se*, although the role of telomere dynamics of other cell populations has not been ruled out.

In conclusion, our results show for the first time in a wild mammal population that adult immune cell telomere length is influenced by early life environmental conditions which individuals experience. That we find no trade-off between natural variation in growth rate

and adult telomere dynamics suggests that either: *i*) natural variation in European badger growth rates do not result in detectable somatic maintenance trade offs (particularly in the absence of marked 'catch-up' growth) or *ii*) immune cell telomeres are an inappropriate tissue to explore growth/somatic maintenance costs. This work strengthens the view that telomere dynamics may provide a mechanistic link between early and late life performance in the wild. Further research to determine the implications reduced adulthood immune cell telomere length are required in order to fully understand the consequences of early life variation in environmental quality on late life declines in performance.

Chapter 4: Pro-inflammatory cytokine response of a wild mammal declines with age but not with immune cell telomere length

Abstract

Senescence, the age-related declines in performance observed in many species, has been suggested to be associated with a decline in immune system function. However longitudinal assessments of how immune function in wild mammals changes with age remain rare, largely owing to a need for large, longitudinal datasets and expensive, species-specific assays. Here we take advantage of a long term epidemiological study in wild European badgers (*Meles meles*) to determine: i) if variations in immune signalling are related to age, ii) what factors influence the rate of any such age-related changes and iii) whether changes are correlated with immune cell telomere length, which is a putative proximate mechanism causing immunosenescence. We find strong support for a within-individual, age-related decline in the response of an important immune signalling cytokine, Interferon-gamma (IFNγ), to *ex vivo* lymphocyte stimulation. Whilst we found no evidence to suggest that sex or disease status influenced this age-related within-individual decline, the IFNγ response was stronger in females than males and decreased with progression through stages of bovine tuberculosis (bTB) infection (bTB negative > bTB positive > bTB excretor). Finally, we show that the magnitude of the IFNγ response is not correlated with immune cell telomere length. This work suggests that age-related declines in immune signalling can occur in wild mammal populations, strengthening the suggested role of the immune system in contributing to senescent phenotypes observed in the wild. However, that the IFNγ response and immune cell telomere length independently declined with age both highlights the complexity of the immune system and the pitfalls of considering single traits in isolation.

Senescence is defined as the physiological deterioration that occurs within-individuals at advanced age (Monaghan *et al.* 2008). Senescence is known to manifest as decreases in survival probability and reproductive output, however the physiological changes giving rise to such deteriorations in performance remain poorly understood. Age-related deterioration in immune system functions that increase susceptibility to infection and disease are termed immunosenescence (Larbi *et al.* 2008) and are thought to contribute to whole organism senescence (Faragher *et al.* 2014). To date, the vast majority of immunosenescence research has been focused on humans and laboratory organisms and evidence from natural populations of non-model organisms living under ecologically realistic conditions is rare (but see Palacios *et al.* 2011; Nussey *et al.* 2012). Thus it remains unclear whether patterns of immunosenescence observed under standardised laboratory conditions are apparent in free-living non-model organisms in natural environments.

Within the field of eco-immunology (Brock, Murdock & Martin 2014), there is increasing recognition that between individual variation in cytokine production may play a major role in explaining the natural variation in immunocompetence observed in the wild (Zimmerman, Bowden & Vogel 2014). Cytokines are the major signalling molecules of the immune system (reviewed in Zimmerman *et al.* 2014). Tight regulation of cytokine production is vital for the activation and propagation of appropriate immune responses (Arai *et al.* 1990). Biomedical research has implicated age-related changes in pro-inflammatory cytokine production in contributing to immunosenescent declines (Wakikawa *et al.* 1999). First, a chronic weak increase in baseline pro-inflammatory cytokine concentration in the absence of an overt infection is known to be a risk factor for mortality and morbidity in elderly humans (also known as 'inflammaging', reviewed by Franceschi & Campisi 2014). Second, research in laboratory mammals and humans has documented a general shift with increasing organism age from pro-inflammatory (Th1) to anti-inflammatory (Th2) cytokine production in response to immune stimulation (Shearer 1997). Whilst support for such shifts is by no means unanimous (Gardner & Murasko 2002), they have been suggested to contribute to the age-related weakening of cell-mediated immunity and the corresponding increase in disease susceptibility in elderly individuals (Shearer 1997). Understanding the factors

influencing cytokine production is therefore crucial to furthering our understanding of immunosenescence.

Life history theory predicts that differences between males and females in their allocation of resources to costly activities such as reproduction and growth at the expense of immunity could drive sex-specific variation in immune defences (Lee 2006) and, by extension, sex differences in rates of immunosenescence (Martin *et al.* 2006). Similarly, infection and disease themselves could also influence the rate of immunosenescence. Accumulated tissue damage arising from inflammation associated with early-life or chronic infection may increase subsequent rates of immunosenescence (Gavazzi & Krause 2002). Thus, longitudinal assessments of cytokine production and its relationship with age, sex and infection/disease status appear vital to understand natural variation in immune responses and any decline in these in later life.

Considerable insight can be gained in the mechanisms underpinning immunosenescence through investigation of the age related changes in multiple components of the immune system as they may *i)* be borne out of a common mechanism or *ii)* have causal impacts on one another (Downs & Dochtermann 2014). One factor thought to be related to the pro-inflammatory cytokine response is immune cell telomere length. Immune cell telomere length is an important parameter of the immune system that has previously been used as a biomarker of immunosenescence (e.g. Bestilny *et al.* 2000). Immune cell telomeres shorten with age in mammals (Haussmann *et al.* 2003; Armanios 2013; Chapter 2) and are a risk factor for age-related disease and death in humans (Fossel 2012). Previous research has invoked two lines of reasoning for linking immune cell telomere lengths and pro-inflammatory cytokine production to immune stimulation. First, strong pro-inflammatory cytokine responses could promote immune cell turnover and the production of reactive oxygen species, both of which are known to reduce immune cell telomere length (O'Donovan *et al.* 2011). Second, immune cell telomere length could constrain the proliferation potential of lymphocytes, resulting in individuals with shorter immune cell telomeres tending to produce weak pro-inflammatory cytokine responses (Jergović *et al.* 2014). Thus far, the majority of studies examining the relationship between pro-inflammatory biomarkers and immune cell telomere length have been cross-sectional in

nature and have yielded mixed results (e.g. Carrero *et al.* 2008; O'Donovan *et al.* 2011; Jergović *et al.* 2014 but see Wong 2014).

Here we take advantage of a diagnostic assay developed to detect infection with bovine tuberculosis (bTB) specifically for use in European badgers (*Meles meles*) and a long-term study of bTB epidemiology in a naturally infected wild badger population. We explore age-related variation in production of the pro-inflammatory cytokine interferon-gamma (IFNγ) following immune stimulation. Production of IFNγ is important as it has a vital role in regulation of both innate and cell-mediated immunity (Savan *et al.* 2009). Its functions include the induction of antiviral enzymes, priming of macrophages and influencing leukocyte movement (Zimmerman *et al.* 2014). Biomedical research has suggested that IFNγ production in response to stimulation decreases with age in senescent cells both *in vitro* (Mbawuike *et al.* 1997; Dagarag *et al.* 2004) and *ex vivo* in elderly humans (Rink, Cakman & Kirchner 1998; Looney *et al.* 2002; Cherukuri *et al.* 2013). However, declines in IFNγ responses with age are by no means ubiquitous; some studies have documented either no change or even increases (e.g. Bandrés *et al.* 2000; Saurwein-Teissl *et al.* 2002). Whilst such effects have been attributed to differences between laboratory model organism strains (Saurwein-Teissl *et al.* 2002), they are also likely due to the cross-sectional nature of many biomedical studies. This highlights the need for longitudinal assessments of the relationship between age and IFNγ responses in natural populations.

We have tested 1) if there is a within-individual age-related change in the magnitude of the pro-inflammatory cytokine response; 2) if sex and/or bTB infection status influence the rate of within-individual change in pro-inflammatory cytokine response; 3) if individuals that show greater pro-inflammatory immune responses also show longer average immune cell telomere lengths; and 4) whether within-individual changes in pro-inflammatory immune response are correlated with within-individual changes in immune cell telomere lengths. In all cases the pro-inflammatory cytokine response is defined as the magnitude of IFNγ produced by whole blood cultured with a mitogen (Pokeweed) known to non-specifically stimulate the proliferation of T and B-cells (Janossy & Greaves 1972). Non-specific stimuli are thought to capture better the responsiveness of an individual's immune system to a

whole spectrum of pathogens, rather than antigen-specific assays which relate to one particular pathogen and its infection history (Jackson *et al.* 2011).

Study Population

All individual badgers were captured as part of a long-term field study at Woodchester Park, Gloucestershire (UK), which is conducted in an 11 km^2 area of woodland and surrounding farmland. The resident badger population has been subject to continuous ecological and epidemiological monitoring since the mid 1970s. In this high density population, badgers live in social groups consisting of up to 33 individuals defending a common territory (Delahay *et al.* 2006a). During the spring of each year the boundaries of each badger social group territory were approximated through the use of bait marking (Delahay et al. 2000). All badgers captured between January 2010 and October 2013 were anaesthetised and identified from unique alpha-numeric tattoo administered at their first capture. The date, location, social group of capture, sex, weight (to nearest 100g) and age class (juveniles < 1 year, adult ≥ 1) were recorded for each individual. Captures of individuals of unknown age (those that were not identified as juveniles at their first capture event) were excluded from this analysis. For individuals captured in their first year of life, age is defined as the number of days elapsed since 20th February which reflects the mid-February peak in births, as exact dates of birth cannot be readily determined (Roper 2010). All individuals captured were between 0.2 and 11.6 years of age.

IFNγ response to immune stimulation

Heparinised whole blood from each individual was subjected to an IFNγ assay (see Dalley *et al.* 2008 for full methodology). The assay involved detection of interferon-gamma (IFNγ) production in response to stimulation by purified protein derivatives of *Mycobacterium avium* and *M. bovis*, known as avian (PPD-A) and bovine (PPD-B) tuberculin, respectively, via an enzyme-linked immunosorbent assay (ELISA). Whilst the assay is typically used for bTB diagnostics, the positive control involves stimulation with pokeweed mitogen (PWM), a nonspecific and generalized activator of T- and B-cells (Janossy & Greaves 1972). Whilst stimulation targets T- and B-cells, the magnitude of IFNγ production in response to a standardised PWM stimulus also includes IFNγ secreted from other cell types recruited during the inflammatory cascade (e.g. neutrophils). IFNγ response magnitude was measured

in optical density (OD) units. As all samples were assayed in duplicate the average magnitude of IFNγ response was used in all analyses. The mean co-efficient of variation between technical replicates was 16.2% (n=960). Average values were subtracted from the background un-stimulated IFNγ score (no template control) to give a baseline corrected IFNγ score which controls for the possibility of increases in baseline IFNγ concentration due to inflammaging. For full complete methodology see (Dalley *et al.* 2008).

Age

To ensure that our statistical assessment of within-individual variation in IFNγ response with age was not confounded by between-individual effects (e.g. selective disappearance of animals), we applied a within-subject centring approach, following van de Pol & Wright (2009). Variation in age was partitioned into (i) an individual's 'mean age' across all samples collected for that individual, and (ii) its 'Δ age', i.e. the offset of its age at the focal sampling point from its mean age, the effect of which will reflect within-individual changes in IFNγ response with age.

Body Condition

We tested for a relationship between body condition and IFNγ response in order to ensure that any age effects were not being driven by a relationship between cytokine production and age-related changes in body condition (Adams *et al.* 2009). Body condition was estimated using the Scaled Mass Index (SMI: Peig and Green 2010) to account for the fact that variance in body mass increases with length and that males are on average larger than females. The SMI has been found to capture variation in fat and protein reserves more effectively than traditional residual body condition indices (Peig and Green 2009).

Current Disease Status

An individual's bTB infection status was assessed at each capture event using a combination of three diagnostic tests: i) Interferon-gamma (IFNγ), an enzyme immunoassay assessing relative lymphocyte responsiveness to avian and bovine tuberculin; ii) STAT-PAK® (Chembio Diagnostic Systems, Inc), a lateral-flow immunoassay assessing the presence of antibodies to bTB and; iii) microbiological culture of clinical samples (i.e. sputum, faeces, urine and swabs

of wounds or abscesses) to detect *Mycobacterium bovis* (the causative agent of bTB) (see Chambers *et al.* 2009 for discussion of test performance). We used a simplified version of the disease classification system discussed in Delahay *et al.* (2013). Briefly, individuals were classed as 'negative' if they had never tested positive for bTB on any test, 'positive' if they had ever tested positive using the STAT-PAK® (Chembio Diagnostic Systems, Inc) or IFNγ tests, or 'excretor' if M. bovis was isolated by culture. This is a one-way progressive system where by individuals can transition to elevated disease classifications when appropriate tests are positive, but can never return to lower classifications. Of the 960 captures, 480 were classed as 'negative', 432 as 'positive' and 48 as 'excretor'. A total of 52 individuals transitioned between disease categories during the study period, 42 transitioned from 'negative' to 'positive', four from 'positive' to 'excretor' and five from 'negative' to 'excretor'.

Univariate modelling

In order to determine if the IFNγ response declines with increasing age, we implemented a multi-model inference approach (Burnham & Anderson 2002) using linear mixed-models. A total of 30 *a priori* candidate models were defined containing additive effects of all candidate explanatory variables (mean age, Δ age, sex, bTB status and body condition) and all biologically relevant two way interactions (see Appendix G for full model table). The response term for all models, the magnitude of the IFNγ response to pokeweed mitogen (OD units), was $\log_{10}$ transformed in order to ensure the normality of model residuals. Models were ranked and simplified according to rules detailed in (Richards *et al.* 2011) then co-efficients were averaged across all models in which they occur using the MuMIn package (Barton 2014). Model-average coefficients of all parameters with support are discussed in terms of their relative 'weights'. To account for repeated measures, heterogeneity between plate runs, and variation in social group territory quality or disease burden (Vicente *et al.* 2007), we included 'individual ID', 'plate ID' and 'social group' as random intercept terms in all models. Goodness-of-fit was assessed through calculating conditional (total variance explained by the best supported model) and marginal (variance explained by fixed effects alone) R^2 formulations (Nakagawa & Schielzeth 2013) and standard residual plot techniques.

Multivariate modelling

In order to determine if: *i)* individuals that show stronger average pro-inflammatory cytokine responses also show longer average immune cell telomere lengths (between-individual covariance) and/or *ii)* within-individual changes in pro-inflammatory cytokine response are correlated with within-individual changes in telomere length from one measurement to the next (within-individual covariance) we used a Bayesian mixed model approach (package MCMCglmm, Markov-chain Monte-Carlo generalized linear mixed models; Hadfield 2010), in R (R Core Team 2013). The advantage of this approach is that it allows examination of the posterior correlation (and its corresponding confidence interval) between the magnitude of IFNγ production and immune cell telomere length whilst controlling for both fixed (e.g. sex and age) and random factors (e.g. assay plate) which could influence the apparent relationship between traits.

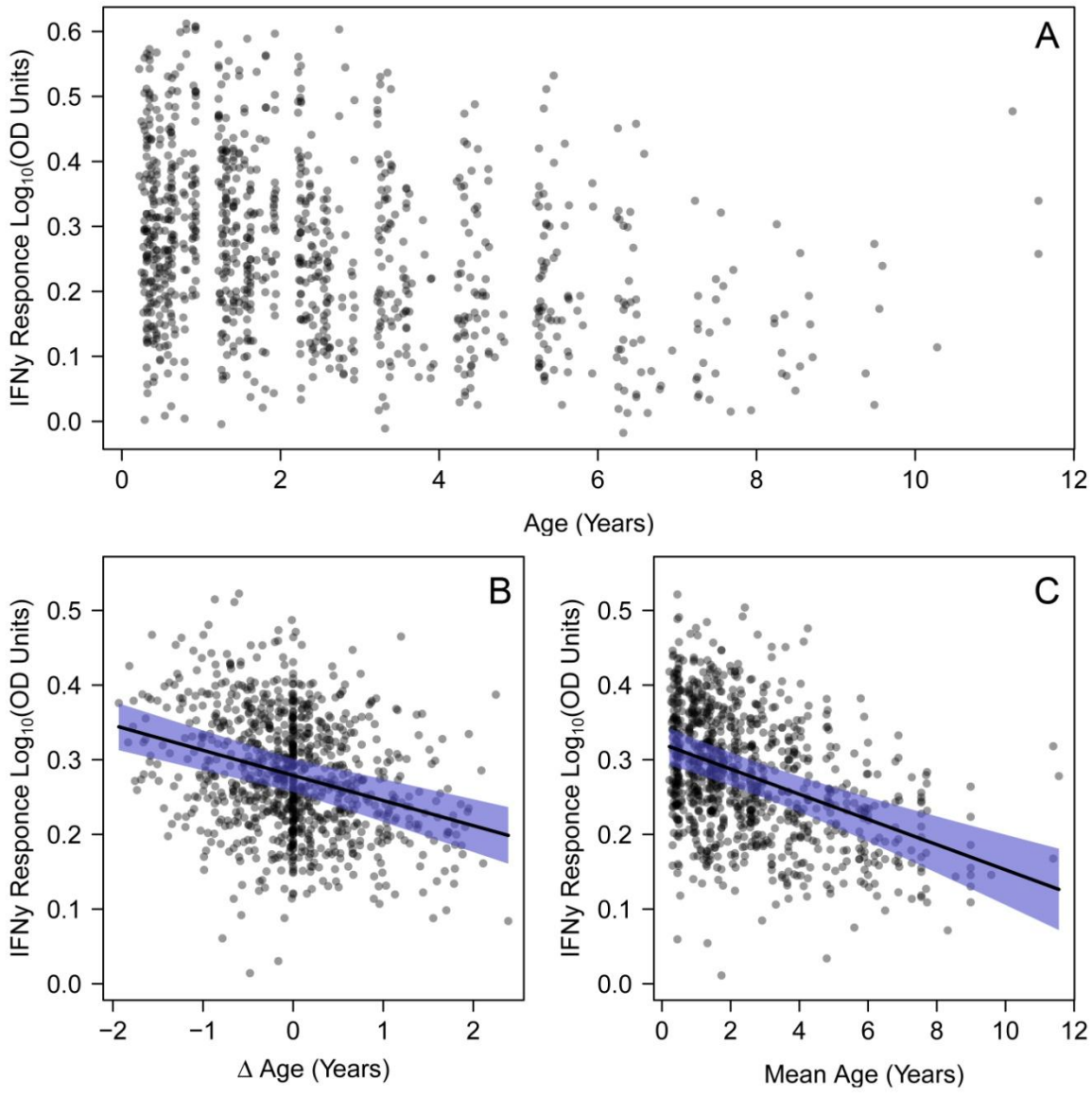
We fitted a bivariate response model with IFNγ response magnitude (960 observations from 295 individuals) and absolute telomere length (360 observations from 172 individuals) as response terms. Both traits were Gaussian distributed. IFNγ was modelled as a function of partitioned age (mean age and Δ age), bTB status and sex (as in the top model as revealed by the univariate modelling process). Absolute telomere length was modelled as a function of partitioned age and bTB status (Chapter 2). Plate was fitted as a heterogeneous random effect for each trait, and completely parameterized (co)variance matrices for the individual identity random effect were used to allow for covariance between each trait. Inverse gamma priors were used with 350000 iterations, a burn-in interval of 80000 and a thinning interval of 500. Repeatability and posterior correlations for the between- and within-individual covariance between IFNγ production and telomere length were estimated according to the methodology outlined in (Dingemanse & Dochtermann 2013). Support for a relationship between the traits would be represented by a correlation estimate with 95% credible intervals not spanning zero. We then repeated the modelling process with the fixed effects of age removed in order to preclude the possibility that controlling for age was influencing the detection of within- or between-individual covariance between IFNγ response and immune cell telomere length. As the removal of age did not change the result, this is not discussed further.

Examining the relationship between the IFNγ response and un-partitioned age strongly suggested the occurrence of an age-related decline in pro-inflammatory cytokine production (Figure 1A). After partitioning age to separate within- and between-individual effects, we found strong support for a within-individual decline in IFNγ response with age whilst controlling for the effects of sex, bTB status, assay plate and social group (Figure 1B, 1C and Table 1). In order to check that the within-individual declines with age were not being driven by age-related changes in bTB infection status, model selection was repeated with the exclusion of all known infected ('positive' and 'excretor') individuals. Full support for a within-individual decline in IFNγ response remained after exclusion of infected individuals (see Appendix H). That the rate of within-individual decline in telomere length occurred at twice the rate of the between-individual decline suggests that individuals with weak IFNγ responses may be selectively lost from the population (Table 1). We found no support for the rate of within-individual change in IFNγ accelerating or decelerating with increasing age (i.e. no statistical support for an interaction between the Δ age and mean age terms).

Table 1. Model averaged results of univariate modelling of the factors influencing IFN**γ** response magnitude. Where: Σ= relative support for a given parameter, β-estimate = direction and magnitude of a parameters effect, SE = Standard Error, CI = Confidence Interval, * = interaction between two parameters, and terms in brackets = reference level for factors (with addition levels shown below). For unabridged model output see Appendix G.

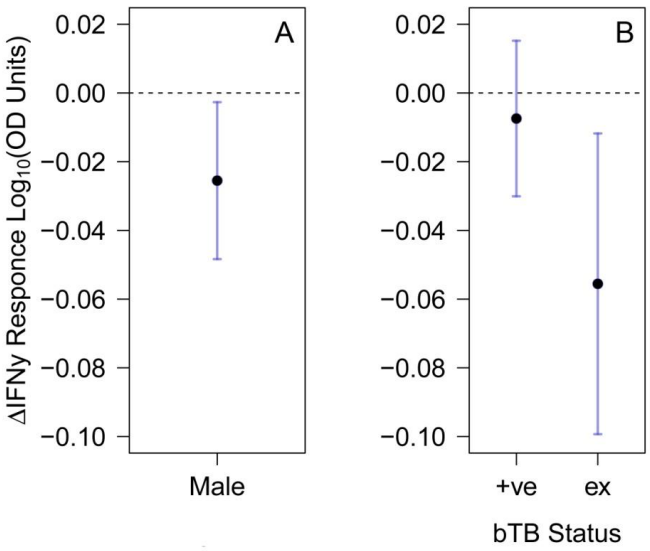
Parameter (Reference Level)	**Σ**	**β**-Estimate	SE	95% CI
Δ Age	1.00	-0.034	0.006	-0.046 to -0.021
Mean Age	1.00	-0.017	0.002	-0.022 to -0.012
Sex (Female)	1.00	-	-	-
- Male	-	-0.026	0.010	-0.046 to -0.005
bTB Status (Negative)	0.81	-	-	-
- Positive	-	-0.007	0.009	-0.026 to 0.011
- Excretor	-	-0.021	0.021	-0.046 to -0.014
Body Condition	0.00	-	-	-
Delta Age * Mean Age	0.00	-	-	-
Delta Age * Sex	0.00	-	-	-
Delta Age * bTB Status	0.00	-	-	-

Figure 1. Relationship between age and IFN**γ** response. (A) Raw data plot of the relationship between IFNγ and un-partitioned age; (B) and (C) relationship between IFNγ and Δ age (within-individual changes in age) and mean age (between-individual changes in age) respectively while controlling for random effects, sex, and bTB infection status (Table 1). The grey points present raw data (A) or residuals (B, C), black lines present model predictions and blue shaded areas present 95% confidence intervals.



We found full support for males possessing weaker IFNγ responses than females (Figure 2A), however there was no evidence to suggest that males or females differed in their rate of decline in IFNγ response with age. We found no evidence to suggest that infection influenced the rate of decline in IFNγ response with age. Support was found for an effect of current bTB infection status on IFNγ responses (Figure 2B) though counter intuitively, perhaps, individuals classified as 'positive' possessed weaker IFNγ responses than those classed as 'negative'. We found strong support for individuals classed as 'excretor' possessing weaker IFNγ responses than either 'negative' or 'positive' classifications. We also found no support for the previously reported link between an individual's current body condition and the pro-inflammatory cytokine response (Adams *et al.* 2009). The best supported univariate model explained 58% of the total variation in the data. The fixed effects account for 12% of the total variation, individual identity as a random effect accounted for 19%, and assay plate for 25% and social group for 3%.

Figure 2. Sex and disease associated variation in IFN**γ** response. (A) Predicted change in IFNγ in males in comparison to females (dashed baseline), (B) Predicted change in IFNγ response due to bTB infection status (dashed baseline = Negative, '+ve' = Positive, 'ex' = Excretor). The black points present model predictions and the blue error bars present 95% confidence intervals.



Both IFNγ response ($Repeatability_{IFN\gamma}$: 0.55; 95% CI: 0.49 to 0.61) and average immune cell telomere length ($Repeatability_{telo}$: 0.39; 95% CI: 0.28 to 0.53) were found to display repeatable between-individual differences through time and both traits show clear within-individual declines with increasing age (Appendix I). Despite this, we found no support for the existence of either a between-individual correlation (r_{ind}: -0.03; 95% CI: -0.18 to 0.18) or within-individual correlation (r_e: -0.01; 95% CI:-0.10 to 0.15) between the two traits after controlling for sex, disease, age and assay plate effects.

The results presented here strongly suggest that age-related declines in expression of a pro-inflammatory cytokine occur in this wild population of mammals. Through longitudinal sampling and an age-partitioning approach we have avoided the commonly confounding elements of many cross-sectional studies of humans and laboratory animals and have shown that the declines observed are occurring at the within-individual level. We found evidence to suggest that male badgers have weaker pro-inflammatory cytokine responses than females, though there was no difference between males and females in the rate of change with age. We also found weak associations between the progression of bovine tuberculosis infection and IFNγ response. Despite putative mechanistic links between immune cell telomere length and the pro-inflammatory immune response, we found no evidence that: i) individuals with strong IFNγ responses also possessed longer immune cell telomeres or ii) that the within-individual change in IFNγ response was correlated with within-individual change in immune cell telomere length. Below, we discuss the potential mechanisms underpinning observed declines in immune system traits with age and their implications for our understanding of senescence in natural populations.

We used an *in vitro* lymphocyte stimulation assay in order to measure how the production of a key pro-inflammatory cytokine alters with increasing age. The assay quantifies the magnitude of IFNγ produced by whole blood after T- and B-cell stimulation with a non-specific mitogen (pokeweed). Our findings suggest that there is a within-individual decline with increasing age in the strength of the pro-inflammatory IFNγ response to this form of immune stimulation. The decline detailed here is consistent with previous work in humans and model organisms demonstrating that weakening IFNγ responses with age contribute to a general shift from pro-inflammatory (Th1) to anti-inflammatory cytokine responses in later life (e.g. Dayan *et al.* 2000). Shifting cytokine expression profiles have also been implicated in mediating thymic involution, thought to be one of the major contributing factors to the immunosenescence phenotype (Gruver, Hudson & Sempowski 2007). It is important to note, however, that as only one pro-inflammatory immune marker was assayed we cannot disentangle immune impairment from immune remodelling (decreasing IFNγ production could be compensated through up regulation of other pro-inflammatory cytokines), or

decreases in IFNγ production occurring in the absence of any shift towards a Th2 response (as in Elsässer-Beile *et al.* 1998). It is also possible that a reduction in pro-inflammatory responsiveness with age could be adaptive, particularly if doing so compensates for the age related increases in baseline inflammatory cytokines that are expected to arise through 'inflammaging' (Franceschi & Campisi 2014). That said, our findings highlight the possibility that weak IFNγ responses appear to carry a cost. Individuals with faster rates of decline or weaker initial responses appear to be selectively disappearing from the population, as the rate of within-individual decline with age was found to occur twice as fast as the between-individual decline.

We found evidence suggesting that males on average show weaker pro-inflammatory cytokine responses than females. This is consistent with previous work demonstrating that male badgers possess weaker IFNγ responses to bTB antigen-specific lymphocyte stimulation (Tomlinson *et al.* 2014), and could underpin the sex-specific susceptibility to, and reduced survival during progression of, bTB in this species (Graham *et al.* 2013; Mcdonald *et al.* 2014). Sex-differences in a variety of immune parameters appear widespread in both humans (McClelland & Smith 2011) and wild vertebrates (Zuk & Stoehr 2002), and may arise through male-biased mortality reducing selection for robust immune defences in males in comparison to females. Sex differences in cytokine production could also arise from the immunosuppressive effects of testosterone in males (Zuk & Stoehr 2002) as testosterone expression has been known to be inversely correlated with plasma cytokine levels (Muehlenbein & Bribiescas 2005).

Our analysis highlighted a weak link between bTB disease progression and the magnitude of the IFNγ response, whereby individuals classed as 'positive' showed a slight reduction in IFNγ response in comparison to uninfected individuals, and individuals classed as 'excretor' showed a large reduction in IFNγ response in comparison to both 'negative' and 'positive' individuals. The pattern is compatible with our current understanding of how tuberculosis infection modulates the pro-/anti-inflammatory immune response in humans: *in vivo* and *in vitro* studies have shown that active tuberculosis infection can result in reduced pro-inflammatory responses (e.g. Lienhardt *et al.* 2002; Raja 2004). It is also consistent with previous work in this badger population, which showed that IFNγ responses to bTB antigen-

specific lymphocyte stimulation declined with increasing time after infection (Tomlinson *et al.* 2014). Using observational datasets such as ours, it is near impossible to distinguish whether the relationship observed here reflects individuals with weak pro-inflammatory responses being pre-disposed to disease progression, or whether weak responses are a direct result of disease progression. However, the prognostic implications of variation in pro-inflammatory cytokine responses deserve further examination in wild populations.

Whilst both immune cell telomere length and magnitude of IFNγ response show evidence of within individual declines with age, we found no evidence for within- or between-individual covariance between the two traits. This suggests that age-related declines in the two immune traits are occurring independently and that multiple and complex mechanisms likely underpin age-related declines in different immune parameters. However, we must also acknowledge that the hypotheses linking immune cell telomere length and pro-inflammatory responses are not mutually exclusive: immune cell telomere length could be constraining the pro-inflammatory immune responses (short telomeres result in weak IFNγ production) whilst a pro-inflammatory phenotype could also be directly causing telomere attrition (strong IFNγ production results in short telomeres). Thus it remains possible that the two processes are occurring concurrently, precluding the detection of a clear directional relationship between each trait. It is also conceivable that immune cell telomere length and IFNγ response are too coarsely estimated in order to detect a directional relationship. Immune cell telomere lengths were estimated from a diverse population of immune cell subtypes which may fluctuate dynamically in their proportions. The magnitude of the pro-inflammatory immune response was estimated through a whole blood ELISA whereby the contributions of immune cell subtypes cannot be ascertained. Hence, it may be necessary to estimate immune cell subtype specific telomere lengths (e.g. Robertson *et al.* 2000) and use intra-cellular staining techniques in order to determine subtype-specific cytokine production (Haberthur *et al.* 2010) to account for variation in immune cell subtypes whilst investigating the relationship between cytokine production and immune cell telomere lengths.

The results presented here demonstrate for the first time that within-individual age-related declines in the pro-inflammatory cytokine response occur in a free-living mammal population. Furthermore, these declines appear to occur independently of immune cell

telomere length, which is a putative proximate mechanism influencing immunosenescence that has previously been shown to decline with age in this population (Chapter 2). Whilst we recognise that the measurement of one or two immune biomarkers will never capture the complex and multifaceted immunosenescent phenotype, our work adds to a growing body of evidence suggesting that the reduced effectiveness of the immune system at advanced ages widely observed in humans and laboratory organisms generalises to wild vertebrate populations. Further work specifically quantifying how such biomarkers contribute to an individual's susceptibility to infection, disease and mortality appears crucial in order to fully understand the fitness consequences and evolutionary implications of age related declines in immune system capability.

Chapter 5: Sex differences in senescence: the role of intra-sexual competition in early adulthood

Abstract

Males and females frequently differ in their rates of ageing, but the origins of these differences are poorly understood. Sex differences in senescence have been hypothesized to arise because investment in intra-sexual reproductive competition entails costs to somatic maintenance, leaving the sex that experiences stronger reproductive competition showing higher rates of senescence. However, evidence that sex differences in senescence are attributable to downstream effects of the intensity of intra-sexual reproductive competition experienced during the lifetime remains elusive. Here we show using a 35 year study of wild European badgers (*Meles meles*), that (i) males show markedly higher body mass senescence rates than females, and (ii) this sex difference is attributable to sex-specific downstream effects of the intensity of intra-sexual competition experienced during early adulthood. Our findings provide rare support for the view that somatic maintenance costs arising from intra-sexual competition can cause both individual variation and sex differences in senescence.

Introduction

Senescence, defined as within-individual physiological deterioration with age, has been detected in a wide variety of natural populations, but the causes of the often marked individual variation in senescence rates remain are poorly understood (Nussey *et al.* 2013). A major source of variation in senescence rates is sex; females frequently have longer lifespans than males in humans and other mammals (Promislow 1992; Clutton-Brock & Isvaran 2007; Maklakov & Lummaa 2013) and recent evidence has highlighted associated sex differences in senescence rates in a range of fitness-related traits, including reproductive success (Reed *et al.* 2008; Nussey *et al.* 2009a), body mass (Tafani *et al.* 2012) and telomere length (Barrett & Richardson 2011). Whilst sex differences in senescence rates are now comparatively well documented, the mechanisms that generate such differences remain poorly understood.

It has been hypothesised that sex differences in senescence rates arise from the differing strength of intra-sexual reproductive competition experienced by males and females (Williams 1957; Kirkwood 1977). Evolutionary approaches to this hypothesis have focussed principally on the implications of the sex difference in mean mortality rates that often accompanies sex differences in the intensity of intra-sexual competition (Williams 1957). The sex that experiences stronger intra-sexual reproductive competition often shows higher mean mortality rates, which may thereby differentially weaken the force of selection against deleterious mutations or antagonistically pleiotropic genes acting in late life in that sex (Andersson 1994), leading to the evolution of faster senescence rates and shorter intrinsic lifespans (Williams 1957). Evidence that males in polygynous species frequently do senesce faster than females (Promislow 1992; Nussey *et al.* 2009a) and that species with stronger degrees of polygyny show more marked sex differences in reproductive lifespan (Clutton-Brock & Isvaran 2007) is therefore broadly consistent with this view. However, support for a central role for sex differences in mortality rates is far from universal (Graves 2007; Reed *et al.* 2008), highlighting the likely importance of other mechanisms.

From a mechanistic perspective, it has also been hypothesised that individuals may suffer resource allocation trade-offs between the expression of competitive morphologies and behaviour and their simultaneous investment in somatic maintenance, which might thereby lead to steeper senescent declines later in life in the sex that experiences stronger intra-sexual competition (Kirkwood 1977; Bonduriansky *et al.* 2008). This perspective highlights the potential limitations of focussing solely on the implications of mortality rates, and predicts instead a direct effect of the intensity of intra-sexual competition experienced during early adulthood on both individual variation in senescence rates and the extent of any sex difference observed in senescence rates (Nussey *et al.* 2013). However, evidence that variation in the intensity of intra-sexual competition experienced predicts individual variation in senescence rates is rare and derives solely from studies of a single sex (Sharp & Clutton-Brock 2011; Lemaître *et al.* 2014), leaving it unclear to what extent sex differences in senescence are also attributable to this same mechanism. Specifically, if somatic maintenance costs arising from intra-sexual competition do contribute to sex differences in senescence rates, one would predict (i) higher rates of senescence in the sex that experiences stronger intra-sexual reproductive competition, and (ii) that the magnitude of any sex difference in senescence rate observed should be predicted by the intensity of intra-sexual competition experienced during early adulthood by members of one or both sexes.

Here we test both predictions using data from a 35 year longitudinal field study of the European badger (*Meles meles*), comprising 13,196 captures of 2409 known-age individuals. We directly compare the age-related body mass trajectories of males and females, and then investigate whether the observed sex difference in body mass senescence rates is attributable in part to downstream effects of intra-sexual competition experienced during early adulthood. Variation in body mass explains considerable variation in both survival and reproductive success in mammals (Gaillard *et al.* 2000) and is recognised as a useful phenotypic indicator of somatic state (Bérubé *et al.* 1999), but the causes of variation in late-life declines in body mass have received remarkably little attention (Mysterud, Solberg & Yoccoz 2005; Nussey *et al.* 2011; Tafani *et al.* 2012). In the European badger, body condition is an important trait underlying reproductive success (Woodroffe & MacDonald 1995; Dugdale, Griffiths & Macdonald 2011a). Senescent declines in body mass might therefore underpin recent observations of reproductive senescence in this species (Dugdale

et al. 2011b). Male European badgers contest mating opportunities both within and among social groups, leading to high rates of extra-group paternity (up to 50% of offspring; Dugdale *et al.* 2007; Annavi *et al.* 2014) and elevated variance in reproductive success among males (Dugdale *et al.* 2007, 2008, 2011b). In high density populations, there is also evidence of reproductive skew among females within a group (Woodroffe & MacDonald 1995; Dugdale *et al.* 2008). Whilst the extent to which intra-sexual competition contributes to reproductive skew and extra-group paternity is not clear (Annavi *et al.* 2014), higher incidence of bite wounding and mortality among males coupled with the clear male-biased sexual size dimorphism in this species, strongly suggests that overt intra-sexual reproductive competition is more intense among males than females (Macdonald *et al.* 2004; Delahay *et al.* 2006b; Graham *et al.* 2013).

Specifically, we test two key predictions: if somatic maintenance costs arising from intra-sexual reproductive competition play a key role in generating sex differences in senescence rates: i) male European badgers should show a faster rate of late-life decline in body mass than females, and ii) this sex difference in senescence rate should be attributable in part to downstream effects of the intensity of intra-sexual reproductive competition experienced in early adulthood. To test the second prediction, we test for sex-specific downstream effects of three proxies for the strength of reproductive competition experienced during an individual's early adulthood (the local densities of adult males and adult females, and the local adult sex ratio) on their rate of body mass senescence later in life, while controlling for any effect of 'current' badger density on their body mass during the senescent period. We also address the possibility of downstream effects on senescence rates of generalised foraging competition in early adulthood (rather than intra-sexual reproductive competition *per se*; following Nussey *et al.* 2007), by also examining the explanatory power in our body mass senescence models of the total badger density experienced during early adulthood. We apply a linear mixed-model approach throughout, which allows us to examine changes in body mass with chronological age whilst controlling for selective disappearance and terminal effects that might otherwise obscure or exaggerate patterns of senescence (Van De Pol & Verhulst 2006).

We utilise data from the long-term Woodchester Park field study (Gloucestershire, UK), where the resident high density European badger population has been continuously monitored since the 1970s (Delahay *et al.* 2000). Each year, the boundaries of all social group territories in the 11km^2 site were approximated by bait marking in spring (Delahay *et al.* 2000), and badgers trapped at all active setts for two nights, four times per year. Badgers were anaesthetised and identified through a unique tattoo administered at first capture. The location, sex, mass (to nearest 100g) and age class (juveniles < 1 year, adult ≥ 1) were recorded for each individual. Captures of individuals of unknown age (those not identified as juveniles at first capture) or unrecorded mass, sex, social group or sett location were excluded from our body mass analyses. Bovine tuberculosis (bTB) infection status was determined for all individuals at every capture (Tomlinson *et al.* 2013). In a detailed investigation of the impact of bTB on badger body mass, Tomlinson *et al.* (2013) identified that individuals begin to lose mass at advanced stages of infection (respiratory or dissemination stages). To avoid such bTB-related body mass losses influencing the body mass dynamics documented here, all captures of individuals at advanced stages of bTB infection were excluded from the analysis. Age is defined throughout as the number of days elapsed since 20th February in their first year of life, which reflects the mid-February peak in births as exact birth dates cannot be readily determined (Roper 2010).

Statistical Analysis

All statistical analyses employed linear mixed-effects models using the "lmer" function of lme4 (Bates 2012) in R Version 2.15.2 (R Core Team 2013). Body mass was used as the response variable in all analyses. Three random intercept terms were included in all models: individual (to account for among-individual variation); social group (to account for heterogeneity in territory quality Delahay *et al.* 2006a); and year of capture (to account for temporal environmental heterogeneity; Macdonald *et al.* 2010).

Selective appearance and disappearance (the non-random arrival in, or departure from, the data set of individuals as age increases) can influence the detection and apparent trajectory

of senescence (Hämäläinen *et al.* 2014). Selective appearance is not an issue in our analyses as all included individuals were caught in their first year of life. We controlled for selective disappearance by including in all models a linear effect of age at last capture (ALC), defined as the age (in days) at which a badger was last captured (Van De Pol & Verhulst 2006). Finally, as terminal effects (a potentially age-independent change in the response term prior to death, due for example to sickness that leads to death) can also complicate the detection and interpretation of age-related patterns (Tafani *et al.* 2012; Froy *et al.* 2013), we included a binary variable reflecting whether or not an individual was in its last year of capture (LYC). To avoid biasing ALC or LYC estimates, all observations from individuals likely to have been alive at the end of the study (those caught in the last two years) were excluded from our analyses.

As the analyses utilised an observational dataset with a large number of candidate explanatory variables and demanded comparisons of nested and non-nested models, we implemented the information theoretic (IT) model selection approach using Akaike's information criterion correcting for small sample size (AICc) (Burnham & Anderson 2002). For each of the senescence modelling sections (A and B below), an *a priori* list of candidate models were defined (see below) and then ranked based on AICc. In the interests of parsimony, more complex models were removed from the analysis if a simpler nested version of that model attracted greater support (a lower AICc) (Richards *et al.* 2011). Following such removals, the remaining models with some support (defined as ΔAICc < 6 from the best supported model) were retained in the top model set. Akaike weights were used to gauge relative support for each model in the top model set, and were defined as the likelihood of a given model divided by the total likelihood of all candidate models in the top model set (Burnham & Anderson 2002). To calculate the total variance explained by the best supported model (fixed plus random effects) and the fixed effects alone we used conditional and marginal R^2 formulations respectively (Nakagawa & Schielzeth 2013). Model fit was assessed using standard residual plot techniques.

Senescence Models

A) Sex differences in late-life body mass dynamics

To determine whether the sexes differ in their age-dependent body mass trajectories in late-life, a set of 21 candidate models (see Appendix J) were defined with body mass (kg) as the response term. We used a quadratic age term to capture the curvilinear relationship between chronological age and body mass. Models that included age (in years) as a categorical fixed factor had no support in comparison to quadratic age models and are therefore not discussed further (ΔAICc = +20.7 relative to the equivalent top quadratic model). In order to preclude the detection of a quadratic age term due to increases in body mass in early life (rather than an accelerating late-life decline), our senescence models only included individuals aged five years and over (the timing of the population-level peak in body mass for both sexes; see Appendix K). This dataset comprised 1241 measures from 297 individuals (107 males, 190 females). In order to test for sex differences in body mass trajectories with age, we assessed the evidence supporting an interaction between sex and both the linear- and squared-age terms that comprise the age-related quadratic. ALC and LYC were included in all models as fixed effects to control for selective disappearance and age-independent terminal changes in body mass respectively (see above). Following Tafani *et al.* (2012) we also allowed for the possibility of a sex-specific terminal decline in body mass, by fitting an interaction between sex and LYC. As previous studies (Macdonald *et al.* 2002; Tafani *et al.* 2012) have reported effects of month-of-year and current social group size (calculated as the number of unique individuals trapped within a group's territory in a given year) on badger body mass, both were included in all models as covariates.

B) The influence of early-adulthood intra-sexual competition on late-life body mass dynamics

In order to determine if the strength of intra-sexual competition in early adulthood influences the rate of body mass decline in late life, we defined early adulthood as the first two years after sexual maturation (between 365 and 1095 days old; a period in which badgers of both sexes accrue significant reproductive success (Dugdale *et al.* 2011b). The first two years of adulthood were used, rather than the entirety of adulthood, in order to (i) investigate the *downstream* effects of reproductive competition on body mass senescence trajectories, while also controlling statistically for effects of *current* competition on body

mass *during* the senescent period; and (ii) reduce the co-linearity between the density metrics used to describe the competitive environment in early adulthood and the equivalent 'current' density metrics calculated during the senescent period.

The modelling process utilised the best supported model from section (A) (in which body mass was dependent on age and an interaction between age and sex, whilst controlling for selective disappearance and terminal effects, current social group size, and month-of-year) and investigated the effects of three additional predictors that provide proxies for the strength of local intra-sexual reproductive competition in early adulthood: adult male density, adult female density and adult sex ratio (see below for calculation methods). Juveniles (<1 year old) were excluded from each as they are unlikely to compete for reproductive opportunities (Dugdale *et al.* 2011b). If investment in reproductive competition during early adulthood does play a key role in determining body mass senescence rates, the body mass senescence rates of males are predicted to increase with increasing early adulthood male density and/or increasingly male-biased early adulthood local sex ratios, while those of females may also increase with increasing early adulthood female density. To verify that any detected effect of early adulthood male or female density could not be attributable instead to a correlated effect of total badger density, due for example to generalised foraging competition (as European badgers show no clear sex difference in foraging niche Robertson *et al.* 2014), we also examined the explanatory power of early adulthood total density (local adult male density + adult female density). To allow for the additional possibility that resource competition as a juvenile impacts rates of senescence (Nussey *et al.* 2007), we also examined the explanatory power of the local population density (local total adult density + local juvenile density) experienced during the juvenile period (0–365 days of age). Finally, having detected apparent downstream effects of early adulthood intra-sexual competition, we investigated whether these could be attributable instead to effects of the (potentially correlated) 'current' life competitive environment *during* the senescence period, by contrasting the explanatory power of the early adulthood and 'current' life versions of the relevant intra-sexual competition metric.

Local Competition Metrics

Local density metrics were estimated by first calculating an annual value for the local density of each class of individuals (adult males, adult females, and juveniles) around each badger sett, defined as the total number of unique badgers of each class caught within a 280m radius of each sett in each year. This search radius provides coverage equal to the mean territory size of a social group across the study period (24.5 hectares), constitutes a likely spatial scale for local competition, and avoids making assumptions about the relative contributions to local competition of within- or extra-group individuals (Dugdale *et al.* 2007). The annual value for local sex ratio (LSR) for each sett was defined as the ratio of local adult male density to total local adult density (adult male density + adult female density). The proxies for the strength of intra-sexual competition in 'early adulthood' (adult male density, adult female density and local sex ratio) for each individual badger were then determined by identifying all of the setts at which it was caught during its second and third years of life, and then averaging the relevant annual values of the focal metric for those setts (when a badger was caught at multiple setts during this period, a weighted average was calculated to account for the number of times that it was caught at each sett). Likewise, the local population density during the 'juvenile period' for each badger was calculated as a weighted average, over all of the setts at which the badger was caught in its first year of life, of the sum of the annual values for local juvenile density and local total adult density. Finally, the 'current' life values for each of the proxies for the strength of intra-sexual competition were considered to be the annual value of that metric calculated for the sett at which the badger was caught when the focal body mass measure was taken.

Males show a faster rate of body mass senescence than females

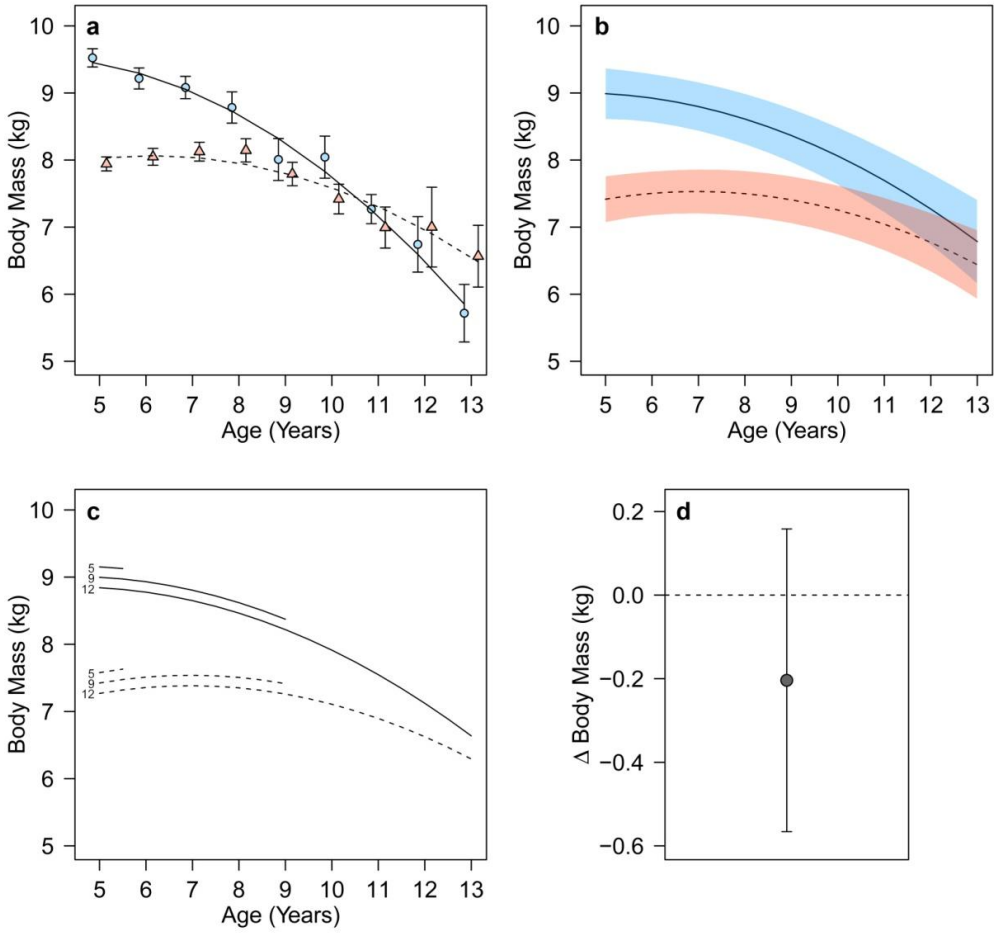
Simple inspection of the raw population-level patterns of body mass change with age after five years of age (when peak body mass is attained; Appendix K), suggests that both sexes show late-life declines in body mass, and that males show steeper late-life declines than females (Figure 1a). The best supported linear mixed-model confirms that this decrease in body mass in late life remains after controlling for the effects of social group, year, month of capture, current social group size and both selective disappearance and terminal effects (Figure 1b; Table 1). The interaction between chronological age and sex indicates that the rate of body mass loss with age among males is indeed higher than that among females, and received full support under model selection (Table 1; Appendix J). This sex difference cannot be attributed to males simply being larger than females (and so having more mass to lose), as repeating the analysis using standardised body condition in place of body mass yielded similar results (Appendix L). The best supported body mass model (Table 1) explained 78.7% of the variation in body mass, of which 42.5% was explained by the fixed effects.

Our models controlled for several additional sources of variation in late-life body mass (Table 1). For each year of increase in Age at Last Capture (ALC) individuals were on average 39g lighter, reflecting the selective disappearance of heavier individuals from the data set within increasing age (Figure 1c). There was also some support for a terminal effect, reflecting an age-independent decrease in body mass of ~200g in an individual's last year of capture (Figure 1d). There was no support for a sex difference in the terminal effect (Table 1). Failure to account for these selective disappearance and terminal effects could therefore have falsely exaggerated apparent late-life declines in body mass. Our models also confirmed and controlled for the previously described negative relationship between social group size and body mass (-22g per group member).

Table 1. Model selection on the factors affecting body mass during the senescent period (≥ 5 years old) after implementation of a model nesting rule. The grey area denotes the models included in the top set, ✓ = terms included in the model, * = interaction between two terms, df = degrees of freedom, AW = adjusted weight after removal of more complex models with less support, SGS = current social group size, ALC = Age at Last Capture, LYC = Last Year of Capture. The top model (in bold) was used as the basis for Figure 1 and as the base model for the analysis of the downstream effects of competition in early adulthood (presented in Table 2). For unabridged model output see Appendix J.

Sex	Month	SGS	ALC	Age	Age^2	Age*Sex	LYC	df	**Δ**AICc	AW
✓	✓	-0.02	-0.04	0.42	-0.03	✓	✓	23	0.0	0.89
✓	✓	-0.02	0.00	0.39	-0.03	✓		22	4.3	0.11
✓	✓	-0.02	-0.04	0.35	-0.03		✓	22	15.9	0.00
✓	✓	-0.02	0.00	0.32	-0.03			21	20.6	0.00
✓	✓	-0.02	-0.03	-0.12			✓	21	30.1	0.00
✓	✓	-0.02	0.01	-0.15				20	34.9	0.00
✓	✓	-0.02	-0.12				✓	20	58.5	0.00
✓	✓	-0.02	-0.07					19	97.1	0.00

Figure 1. Sex differences in body mass senescence. (a) presents mean body mass and standard error for males (blue/circles) and females (red/triangles) for each year of age. Quadratic regression lines were fitted to the means for males (solid) and females (dashed). (b) presents predicted body masses of males (solid line/blue) and females (dashed line/red) with age from the top model in Table 1. Predictions were for badgers outside of their year of last capture, with age at last capture (ALC) and social group size set to their mean values (9.1 and 12.4), and month set to July. The shaded areas present 95% confidence intervals based on fixed effects uncertainty. (c) presents the effect of ALC for males (solid lines) and females (dashed lines) for individuals last caught at ages 5, 9 and 12 years. (d) presents the terminal effect; the predicted change in body mass of individuals in their last year of capture (whiskers present the 95% confidence interval).



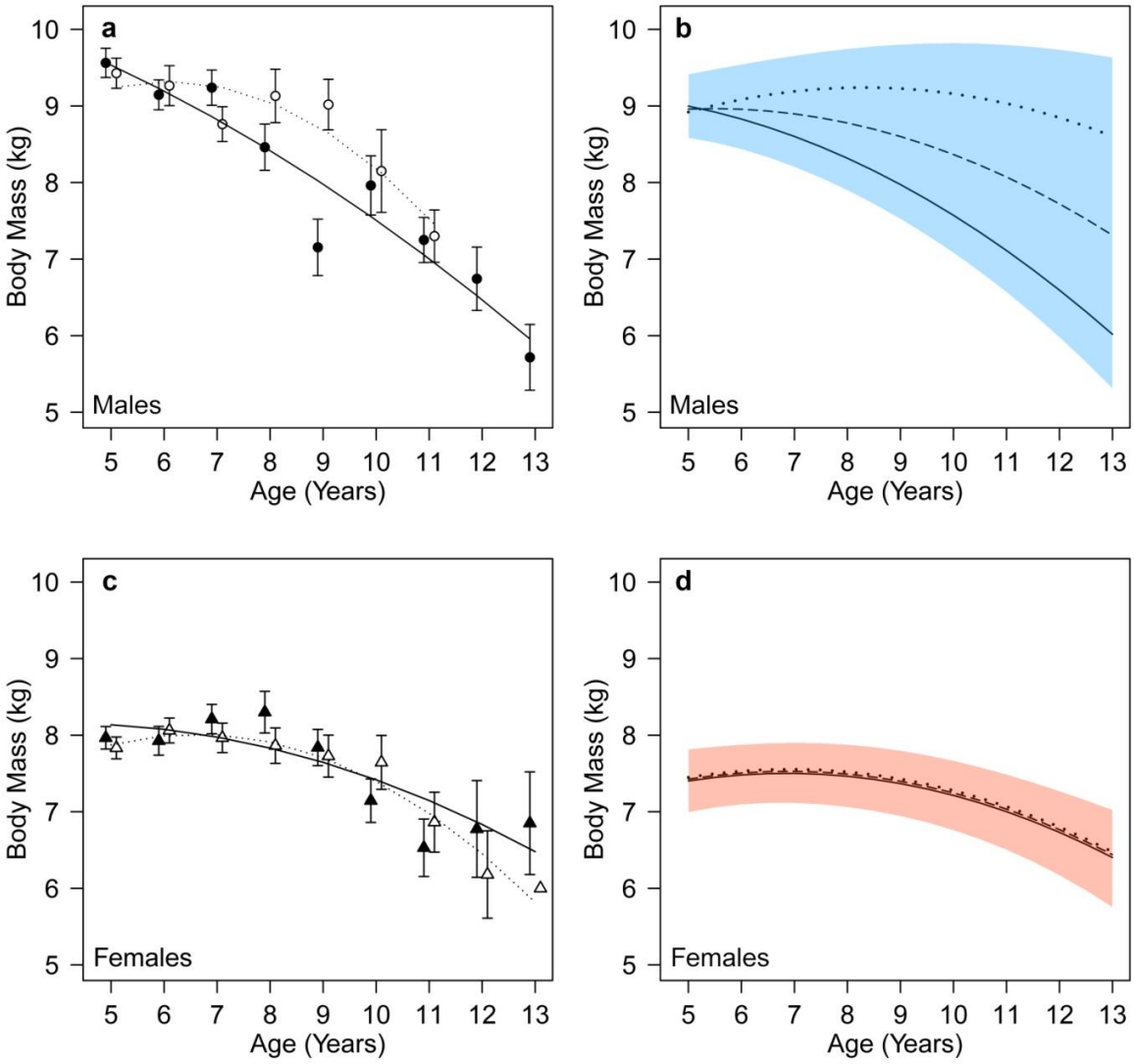
The sex difference in body mass senescence rate is predicted by the local male density that males experienced during early adulthood.

The local adult male density that an individual experienced during early adulthood was found to positively predict its rate of late-life decline in body mass for males but not females (reflected as support for a three way interaction between early adulthood male density, age and sex; Table 2). While this sex-specific response to early adulthood male density is not obvious when simply examining the population-level patterns in the raw body mass data (Figures 2a and 2c), it becomes clear after controlling for the effects identified above of social group, year, month of capture, current social group size, selective disappearance and terminal effects (Figures 2b and 2d; Table 2). Males that experienced higher local male density in the first two years of adulthood showed faster rates of body mass senescence than those that experienced lower local male density (Figure 2b), whereas female body mass senescence rates were unaffected by early adulthood local male density (Figure 2d). The magnitude of the sex difference in body mass senescence rate is therefore predicted by the intensity of intra-sexual competition experienced by males during early adulthood. Indeed, the late-life body mass trajectories of males that experienced low male densities in early adulthood are statistically indistinguishable from those of females (Appendix M), consistent with intra-sexual competition in early adulthood playing a central role in generating the observed population-level sex difference in body mass senescence rates.

Table 2. Model selection on the competitive metrics hypothesised to influence the rate of body mass senescence. The grey area denotes the models included in the top model set. The first three columns represent terms fitted in addition to the base model (see below), where: * = interactions between terms, + = additive terms, df = degrees of freedom, AW = adjust weight after removal of more complex models with less support, EA = Early Adulthood, LSR = Local Sex Ratio, JP Density = local population density during the juvenile period (0 - 365 days old). The base model was defined as: Body mass ~ Month + ALC + Social Group Size + LYC + Age*Sex + Age^2 + (1|ID) + (1|Year) + (1|Social Group); the top model from Table 1.

Base model +					
Age*Sex*____	Age*____	Age+____	df	**Δ**AICc	AW
EA Male Density			27	0.00	0.93
EA Total Density			27	5.23	0.07
	EA Total Density		25	10.69	0.00
	EA Male Density		25	11.22	0.00
BASE MODEL without any additional density terms			23	11.47	0.00
		EA Male Density	24	12.35	0.00
		EA Total Density	24	12.41	0.00
		EA LSR	24	12.44	0.00
EA Female Density			27	13.33	0.00
		EA Female Density	24	13.48	0.00
		JP Density	24	13.55	0.00
EA LSR			27	13.89	0.00
	EA LSR		25	14.07	0.00
	JP Density		25	14.29	0.00
	EA Female Density		25	14.41	0.00
JP Density			27	14.48	0.00

Figure 2. The effect of early adulthood male density on late-life body mass. (a) and (c) present raw means and standard errors for each year of age, with quadratic regression lines fitted through the means, for individuals experiencing above (filled points/solid line) or below (open points/dotted line) mean local male density. (b) and (d) present the predicted relationship between age, the local male density experienced in early adulthood and body mass, from the best supported model in Table 2. Dotted line = low density (1.7 males per 24.5 hectares); dashed line = average (4.1); solid line = high (6.5). Predictions represent badgers outside of their last year of capture, with age at last capture and social group size set to their mean values (9.2 and 12.7 respectively), and month set to July. The upper and lower limits of each shaded area represent 95% confidence interval estimates based on fixed effects uncertainty.



Direct comparison of the explanatory power of the three proxies for the strength of intra-sexual reproductive competition in early adulthood indicated that early adulthood male density had strong support as a predictor (93% of adjusted model weight), while early adulthood female density and local sex ratio had none (Table 2). The weight of evidence also suggests that this apparent downstream effect of male density in early adulthood is not simply a by-product of a correlation between male density and total adult density in early adulthood, as male density attracted substantially stronger support (Table 2). Likewise, there was no support for a downstream effect of the local population density experienced during the juvenile period on body mass later in life (Table 2). Finally, the apparent downstream effect of male density in early adulthood also cannot be readily attributed to a correlated direct effect of the 'current' male density experienced *during* the senescent period, as comparing the explanatory power of the two predictors yielded overwhelming support for a role for early adulthood male density (Table 3).

Table 3. Direct comparison of early adulthood male density and current male density. Direct comparison of interactions between age and male density experienced in early adulthood ('EA Male Density') or male density experienced during the senescent period ('Current Male Density') revealed full support for a sex-specific effect on body mass senescence rates of the male density experienced during early adulthood (Age*Sex*EA Male Density). The grey area denotes the models included in the top model set. The first three columns represent terms in addition to the base model (see below), where: * = interactions between terms, + = additive terms, df = degrees of freedom, AW = adjusted weight after removal of more complex models with less support. The base model was defined as: Body mass ~ Month + ALC + Social Group Size + LYC + Age*Sex + Age^2 + (1|ID) + (1|Year) + (1|Social Group); the top model from Table 1.

Base model +					
Age*Sex*____	Age*____	Age+____	df	**Δ**AICc	AW
EA Male Density			27	0.0	1
	EA Male Density		25	11.2	0
		EA Male Density	24	12.4	0
		Current Male Density	24	12.9	0
	Current Male Density		25	14.5	0
Current Male Density			27	18.1	0

Our longitudinal analyses reveal a clear sex difference in the rate of late-life decline in body mass in European badgers; males show faster rates of decline than females. As body condition is a predictor of reproductive success in this species (Woodroffe & MacDonald 1995; Dugdale *et al.* 2011a), steeper body mass senescence among males than females provides one likely explanation for the steeper late-life declines in reproductive success recently documented among male European badgers (Dugdale *et al.* 2011b). As overt intra-sexual reproductive competition appears to be more intense among male European badgers than females, the observed sex difference in late-life body mass dynamics is consistent with a role for intra-sexual reproductive competition in generating sex differences in senescence rates, and echoes previous reports of higher senescence rates among males in polygynous species (Clutton-Brock & Isvaran 2007; Nussey *et al.* 2009a; Lemaître *et al.* 2014). Uniquely, however, our findings also suggest that individual variation in the intensity of intra-sexual competition experienced in early adulthood generates downstream variation in body mass senescence rates among males but not females, and hence influences the extent of the sex difference observed in body mass senescence rates. As such, our findings provide rare support for the view that sex differences in senescence are not simply an evolved consequence of sex differences in mortality rates, but arise in part from downstream effects of intra-sexual competition experienced during the lifetime. Indeed, that the late-life body mass trajectories of males that experienced low male densities in early adulthood were statistically indistinguishable from those of females, highlights the possibility that the sex difference in body mass senescence documented here may be attributable in its entirety to downstream effects of intra-sexual competition.

Multiple lines of evidence suggest that intra-sexual reproductive competition is more intense among male European badgers than females; males contest matings within and between groups, show higher rates of bite-wounding and mortality than females, and the species shows male-biased sexual size dimorphism (Macdonald *et al.* 2004; Delahay *et al.* 2006b; Graham *et al.* 2013). As such, the observed sex difference in mean body mass senescence rates is compatible with both prevailing theories that invoke a key role for intra-sexual reproductive competition in generating sex differences in senescence. First, classical

evolutionary theory predicts that steeper senescent declines should arise among males in polygynous species because the higher male mortality rates that often accompany polygyny are expected to differentially weaken selection among males against deleterious mutations or antagonistically pleiotropic genes acting in late life (Williams 1957). Second, mechanistic approaches also predict steeper senescent declines in the sex that experiences stronger intra-sexual reproductive competition, due to somatic maintenance costs arising from their differential investment in competitive morphologies and behaviour (Kirkwood 1977; Bonduriansky *et al.* 2008).

However, our results also suggest that both the individual variation and sex difference in late-life body mass dynamics in this population arise in part from downstream effects of the intensity of intra-sexual competition experienced in early adulthood. A direct comparison of the sexes revealed strong support for a positive effect of early adulthood local male density on the rate of body mass senescence in males, but not females. This apparent downstream effect of the intensity of intra-sexual competition experienced *within* a male's lifetime is important, as it is not predicted by classical evolutionary approaches that consider sex differences in senescence solely a product of sex differences in mean mortality schedules (Lemaître *et al.* 2014). Such downstream effects of intra-sexual competition are, however, predicted if sex differences in senescence are envisaged to arise from a trade-off between investment in reproductive competition and somatic maintenance (Kirkwood 1977; Bonduriansky *et al.* 2008). Indeed, if such a trade-off in males played a central role in generating sex differences in senescence rate, male badgers experiencing low levels of intra-sexual competition in early adulthood would be predicted to show senescence rates akin to the population average for females, which is precisely what we found (compare Figures 2b and 2d; and see Appendix M). As such, our findings provide some of the strongest support to date for the hypothesis that sex differences in senescence rate arise from somatic maintenance costs entailed in intra-sexual competition.

We were able to evaluate several potential alternative explanations for our finding that male density in early adulthood predicts the rate of late-life decline in body mass among males. First, this finding cannot be attributed instead to a downstream effect of *total* adult density in early adulthood (e.g. due to density-dependent somatic maintenance costs to all

individuals Nussey *et al.* 2007), as our models revealed substantially stronger support for an effect of early adulthood *male* density than early adulthood *total* density (Table 2). Nor can this finding be attributed instead to a direct effect on a male's late-life body mass of the male density experienced *during* the senescence period (potentially correlated with male density during early adulthood), as competing models revealed full support for an effect of *early adulthood* male density and no support for a comparable effect of *current* male density (Table 3). Likewise, all of our models control for the previously documented effects of social group size on a badger's current body mass (Rogers, Cheeseman & Langton 1997; Macdonald *et al.* 2002). As our models also control for both selective disappearance and terminal effects, it is also unlikely that either process is leading here to an overestimation of the late-life declines in body mass.

That the local adult sex ratio experienced in early adulthood had no detectable effect on late-life body mass declines in either sex is not unexpected, as local adult male density may provide a stronger proxy for the strength of intra-sexual reproductive competition among male European badgers than local sex ratio for two reasons. First, as females can exhibit asynchronous oestrus over a long reproductive season (with births seasonally synchronised by delayed implantation), the strength of male-male competition for matings at any one time may be largely unrelated to the local density of females. Second, as only a subset of adult females within a group successfully breed (Woodroffe & MacDonald 1995; Dugdale *et al.* 2008), competition for just this subset may again leave the intensity of reproductive competition among males unrelated to local female density.

Given recent interest in the evolutionary consequences of female-female competition (Stockley & Campbell 2013), it is notable that we found no detectable effect of early adulthood female density on the late-life body mass dynamics of females. While female European badgers frequently do show variance in reproductive success within groups (Woodroffe & MacDonald 1995; Dugdale *et al.* 2008), the extent to which this arises from overt physical contests is unclear. Certainly, lower frequency bite-wounding and lower mortality among females, coupled with male-biased sexual dimorphism, are consistent with intra-sexual competition being less intense among females, and this alone could explain the lack of an evident downstream effect of early adulthood female density. Such patterns also

accord with recent suggestions that females may be more likely than males to resolve conflict without escalated physical contests (Cant & Young 2013); a pattern that could itself weaken intra-sexual selection among females for investment in competitive traits (Young & Bennett 2013) and, by extension, any associated costs of doing so for late-life performance.

Together, our findings provide rare support for the view that sex differences in senescence rates arise from downstream effects of trade-offs between investment in intra-sexual reproductive competition and somatic maintenance (Kirkwood 1977; Bonduriansky *et al.* 2008), and highlight that such trade-offs may shape both inter-individual differences and sex differences in senescence rates in wild populations. Indeed, that the observed sex difference in late-life body mass trajectories is all but absent in individuals that experienced low male densities in early adulthood, suggests that such somatic maintenance trade-offs alone may be sufficient to account for the sex difference in body mass senescence rate documented here. That trade-offs of this kind might also account for the sex differences in senescence documented in other polygynous species (Promislow 1992; Nussey *et al.* 2009a; Lemaître *et al.* 2014), as well as inter-specific variation in their magnitudes (Clutton-Brock & Isvaran 2007), highlights the need for caution when attributing such patterns to evolutionary responses to sex differences in mean mortality rates. Our findings suggest that attempts to understand the origins of sex differences in senescence could now be well-served by focussing on the behavioural and molecular mechanisms that may generate trade-offs between exposure to intra-sexual competition during the lifetime and aspects of late-life performance.

Chapter 6: General Discussion

As overwhelming evidence of senescence has now been documented in the natural populations of many taxa (Nussey *et al.* 2013) we are moving away from simply documenting the existence of senescence in the wild. Research is now shifting towards understanding the fundamental mechanisms underpinning senescence and determining which environmental factors give rise to the considerable variation in its rate and onset observed in nature (Monaghan *et al.* 2008). To that end, I have examined two key traits implicated in influencing the decreases in survival probability and reproductive output in late-life: reduced somatic telomere length and declining immunocompetence. I found evidence of within-individual age-related changes consistent with senescence in both. I also explored the factors (e.g. disease and/or early life environmental conditions) that give rise to variation in the rate of age-related declines in each trait. Finally, I took advantage of a remarkable longitudinal dataset to explore how intra-sexual competition in early adulthood can influence sex-specific body mass senescence rates and give rise to inter-individual variation in senescence trajectories. Below, I discuss the key findings of this thesis, their implications for our understanding of senescence and, where appropriate, suggest future research objectives in light of my findings.

Telomeres in Ecology and Evolution

Much of what we know about telomere length and telomere dynamics in the wild has come from studies of wild populations of birds (e.g. Hall *et al.* 2004; Foote *et al.* 2011; Geiger *et al.* 2011; Heidinger *et al.* 2012; Young *et al.* 2013). The narrow taxonomic scope of current research likely limits the generality of the findings reported to date and highlights a need to examine telomere dynamics in natural populations of other taxonomic groups, such as the mammals. In Chapter 2, I investigated age-related variation in immune cell telomere length of the European Badger and, consistent with the majority of the currently available avian literature (e.g. Juola *et al.* 2006; Barrett *et al.* 2012), found strong support for within-

individual declines in telomere length with age. This suggests that telomere length may also be a crucial determinant of life expectancy in natural mammalian populations.

The key difference in assaying telomere length from the blood samples of mammals and birds is that bird red blood cells are nucleated whereas those of mammals are not (Nussey *et al.* 2014). Mammalian telomere length assays using blood are therefore targeting cells of the immune system (e.g. neutrophils and lymphocytes), whereas comparable analyses in birds will principally be documenting changes in erythrocyte telomere lengths. Examination of the telomere length of immune cells can undoubtedly bring novel immune perspectives to the fields of ecology and evolution (see 'Immunosenescence' below), however caution must be exercised when making direct comparisons with erythrocyte telomere dynamics (or other cell types for that matter). Firstly, as opposed to other somatic tissues it is possible that telomere length could increase with age in immune cells, as telomerase is known to be active throughout the lifespan in human immune cells (Chan & Blackburn 2004; Aubert & Lansdorp 2008). Telomere length restoration through telomerase activity may therefore make immune cell telomere length a less reliable indicator of general somatic cell telomere lengths across the body than the erythrocyte telomere length measures commonly used in the avian literature. Telomerase action could also explain why in Chapter 2, after controlling for the affects of age there was some support for bTB-associated increases in immune cell telomere length ('exposed' individuals had longer telomeres than 'negative' and 'excretors'). The divergent replicative histories and maturational steps involved in generating immune cells also makes generalisations about their telomere dynamics difficult even in the absence of telomerase activity. That said the majority of immune cells within the European badger are composed of neutrophils (~80%) which share many of the properties that make erythrocytes amenable to study telomere dynamics (high turnover and low telomerase activity; Nussey *et al.* 2014). As such, studying the mean telomere lengths of the overall immune cell population (as here) might ultimately give just as valid an indication of general somatic telomere length dynamics as studies of erythrocyte populations. Clearly, future studies seeking to understand the causes and consequences of variation in the telomere lengths of immune cells *per se* could benefit from: *i*) determining the relative proportions of immune cell subtypes contributing to their sample or, *ii*) separating immune cell subtypes (e.g. using a cell-sorter) and determining subtype-specific immune cell telomere length (e.g.

Lin *et al.* 2010), and *iii*) assaying for telomerase activity within each sample (e.g. Haussmann *et al.* 2007). While all such recommendations are possible to implement in studies of humans and the subset of model organisms for which the necessary species-specific techniques have been developed, they are currently beyond the scope of most ecological studies of non-model organisms.

Despite some concerns about the use of immune cell telomere length as a proxy for general somatic telomere length (Nussey *et al.* 2014), in Chapter 3 I showed individuals experiencing harsh early life environmental conditions had shorter immune cell telomere lengths. While similar results have previously been reported from bird erythrocytes, this is the first result of its kind for a mammal, and using immune cell telomere lengths. This supports the assertion that immune cell telomere length can still be a valuable metric for studies in ecology and evolution seeking to understand the impact on somatic maintenance of stress and life-history choices at the individual level. Whilst we only examined the role of environmental stress and growth parameters in influencing adult telomere length, future evolutionary ecological studies looking to understand how variation in telomere length arises and its consequences for individual fitness should also consider the role of genetic inheritance. Understanding heritability is crucial to understanding the selection pressures operating on telomere length and the consequences of variation in telomere length on life history trajectories (Reichert *et al.* 2014). Unfortunately, it was not possible to determine the heritability of immune cell telomere length in this study system as pedigree information is not currently available. Once the pedigree is resolved for this population this will represent an interesting future research objective, particularly as while it has been demonstrated that telomere length is heritable, whether it is maternally or paternally inherited remains unclear (Horn *et al.* 2011; Olsson *et al.* 2011; Eisenberg 2014).

Immunosenescence

Longitudinal assessments of vertebrate immune function particularly of mammals remain rare in the wild (Palacios *et al.* 2007; Nussey *et al.* 2012). This thesis addressed this shortfall by examining the within-individual patterns of age-related change in two immune traits,

immune cell telomere length and pro-inflammatory cytokine response, and found changes consistent with senescence in each (see Chapters 2 and 4). I address each trait in turn below:

Immune cell telomere length has long been used in the biomedical sciences as a biomarker of age-related disease and reduced immune cell replicative capacity arising from the accumulation of critically short telomeres (Fossel 2012). Consequently, immune cell telomere shortening is thought to contribute to immunosenescence (Flores *et al.* 2006; Effros 2011; Fossel 2012). However it was unclear if such declines occurred in natural populations of wild vertebrates. Consistent with current biomedical research, in Chapter 2 I showed that age-related declines in immune cell telomere length occur in the wild. I must also acknowledge that the fitness implications of having short immune cell telomere lengths in this population are not yet known. Whether individuals with short telomere lengths are more susceptible to disease progression or mortality remain open questions. Investigating whether short immune cell telomere lengths are associated with reduced survival and/or increased disease susceptibility will require following the focal individuals sampled here as they transition through disease states throughout their lifetime. Such information may become available with time, however, as the long-term Woodchester park study continues to monitor the fates of the focal individuals whose telomere lengths were assessed in this thesis.

Pro-inflammatory cytokines are vital cell signalling molecules that are required for the generation of robust and appropriate cell mediated immune responses (Shearer 1997). As for immune cell telomere length, biomedical research had implicated age-related declines in pro-inflammatory cytokine production in response to immune stimulation as a factor contributing to immunosenescence, but whether such declines also occurred in natural vertebrate populations was unknown. In Chapter 4, I showed that age-related declines in the pro-inflammatory cytokine (IFNγ) response to mitogenic stimulation do occur in a wild mammalian population. Akin to the immune cell telomere work, I must also acknowledge that the implications of declining pro-inflammatory immune responsiveness for disease susceptibility and mortality remain unknown. Incorporation of pro-inflammatory cytokine response as a time varying covariate into mark-recapture studies of both survival and

disease transmission (e.g. Graham *et al.* 2013) within this population could be highly informative in this respect and this is something that I would be keen to pursue in future. The logic underpinning Chapter 4 makes the assumption that a larger pro-inflammatory cytokine response is always better, but, as I acknowledge, this is not necessarily the case. A highly pro-inflammatory cytokine profile early in life could have deleterious effects later in life through the aggravation of early life degenerative processes which have late life consequences in an antagonistic pleiotropic fashion (Wikby *et al.* 2005). It is also possible that the age-related decline in pro-inflammatory immune response detected reflects adaptive immune remodelling with age rather than immunosenescence (Faragher *et al.* 2014). For example, down regulation of the pro-inflammatory immune response could actually protect individuals from the effects of 'inflammaging' (chronic background inflammation which is not associated with an overt disease state) in late life and actually confer a survival advantage. In order to understand whether individuals in this population exhibit the inflammaging phenomena (Franceschi & Campisi 2014), an assay sensitive enough to detect meaningful variation in baseline circulating cytokine levels must first be developed. There is pervasive evidence in the biomedical literature linking inflammaging with senescence phenotypes, including immunosenescence and declines in cognitive health and energy production (Franceschi & Campisi 2014).

In the case of both of the immune traits studied, I only considered the influence of *current* bTB infection status on their absolute magnitude or rates of decline. Simply assessing the influence of current bTB status on immune cell telomere length could be an over simplification for two reasons. First, it may be that it is *previous* infection history rather than current infection status which has implications for rates of senescence. For example, if chronic infection resulted in more rapid immune senescence in late life via cumulative antigenic load and immune exhaustion (De Martinis *et al.* 2005), the amount of time an individual has spent in a given disease state may be the most informative parameter. Second, there is increasing awareness that studying single infections in isolation can be too simplistic, as natural populations are exposed to multiple pathogens which may interact positively or negatively within their hosts (Telfer *et al.* 2010). European badgers are subjected to multiple pathogens in addition to bTB such as: mustelid herpes virus, trypanosomes and gastro-intestinal parasites (Sin *et al.* 2014). Incorporation of multiple

infections and their co-infection dynamics (Telfer *et al.* 2010) may better explain the inter-individual variation observed in both immune cell telomere length and pro-inflammatory cytokine response. Furthermore, incorporation of further traits (e.g. reproductive effort) across an individual's lifespan could also shed light on resource allocation trade-offs between immunity, somatic maintenance and other life history traits. For example, my last chapter highlights the likely influence of early life exposure to intra-sexual competition on patterns of somatic maintenance, raising the possibility that this too might impact the age-related trajectories of components of immunity.

Variation in Senescence Rates

There is considerable interest within the fields of ecology and evolution in the factors that generate intra-specific variation in senescence rates (Monaghan *et al.* 2008). In Chapter 2 and Chapter 4, I showed that marked and repeatable inter-individual differences exist in immune cell telomere length and pro-inflammatory cytokine response. In Chapter 3, I demonstrated that inter-individual variation in adult telomere length can be influenced by the environmental conditions experienced in early development, variation that might presumably influence the age of onset and rate of progression of senescence in late life. In Chapter 5, I addressed a widespread source of inter-individual variation in the patterns of senescence; differences between the sexes. I found that the magnitude of the sex difference in body mass senescence rate at the population level is dependent on the scale of intra-sexual competition experienced by males in early adulthood. Males that experienced low intra-sexual competition in early adulthood had a senescence rate that was statistically indistinguishable from females, whereas those experiencing high intra-sexual competition show markedly more rapid senescent declines than females. This work lends support to the life history theories of aging, particularly that of the disposable soma theory (Kirkwood 1977). The disposable soma theory hypothesises senescence arises as the result of a trade-off between investment in somatic maintenance and other costly activities (such as immunity). Such trade-offs occur within an individuals lifespan, and thus the disposable soma theory helps us understand why such marked intra-specific variation in senescence rates is observed in nature (Nussey *et al.* 2013; Lemaître *et al.* 2014). Taking this research to

the next level would require two key extensions. Firstly, resolution of a pedigree would both facilitate incorporation of reproductive traits (such as age at first reproduction) which will likely be highly influential in understanding life-history tradeoffs, and facilitate the examination of whether the traits examined in this thesis and their rates of decline with age, are heritable. Further understanding of heritability is crucial if we are to gain a better understanding of the evolution of senescence in natural populations. Secondly, it is an absolute priority to determine whether the age-related traits examined in this thesis are costly. For example, if age-related declines in pro-inflammatory cytokine response do not increase an individual's susceptibility to infection and disease, then it may actually represent adaptive remodelling of the immune system with increasing age rather than senescence.

Concluding remarks

The study of the mechanisms that underpin senescence and the factors that influence variation in its time of onset and rates of progression is still in its infancy within natural populations, particularly of wild mammals. This research has begun to address this knowledge gap and yielded several novel insights. I have provided the first robust assessments of within-individual changes in immune cell telomere length and pro-inflammatory cytokine response in a wild vertebrate population, and found strong evidence consistent with senescence in both parameters. My research also highlights the value of considering how natural variation in the environmental conditions (biotic or abiotic) experienced in early life could generate variation in senescence rates between the sexes and cohorts, but also by extension, between populations and species. There is clearly much still to be elucidated about the mechanisms underpinning senescent declines, and studies investigating wild populations experiencing natural environmental conditions have a key part to play in this. Throughout the course of this PhD I have been constantly struck by the rapid development of assays with the potential to study senescence in wild populations, from next generation sequencing (Magalhães, Finch & Janssens 2010) to the application of demanding biomedical techniques (e.g. Nussey *et al.* 2012). Whilst the development of such techniques to study senescence in the wild is exciting, a balance must be struck between their relevance and feasibility. Undoubtedly, much remains to be learnt from studying the evolutionary ecology of senescence in natural populations, and as such it represents an

exciting field to be involved in. The future integration of mechanistic and evolutionary approaches, in both controlled environments and in the field will be challenging, but vital in order to answer the huge question of why senescence occurs.

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Appendices

Sample acquisition

A 4ml heparinised blood sample was obtained by venipuncture from each captured badger and spun at 3000 rpm for 10 minutes within 30 minutes of the sample being taken. A 40µl aliquot of the resultant buffy coat (immune cell layer) was stored at -80°C until DNA extraction. Buffy coat samples were gently thawed on ice then DNA was extracted using Fermentas Whole Blood DNA extraction spin columns according to the manufacturer's protocol. DNA was eluted in 100µl of low EDTA TE buffer and stored at -20°C until qPCR analysis. Average DNA yield (ng/ul) was 57.1 (SD±29) and average DNA purity (A_{260}/A_{280}) was 1.83 (SD±0.16). DNA integrity was validated by electrophoresis on a 0.7% agarose gel. No evidence of sample degradation was detected.

Relative telomere length qPCR method

In order to measure mean immune cell telomeres in the European badger we used a quantitative PCR (qPCR) approach. This assay has advantages and disadvantages over other methods available which are reviewed comprehensively in (Aviv *et al.* 2011; Aubert *et al.* 2012; Foote *et al.* 2013; Nussey *et al.* 2014) and are not discussed further. Briefly, two sets of primers are designed: one to target repeat sequences associated with telomeric regions (TTAGGG) and a second to a non-variable copy number control gene (we used inter-photoreceptor retinoid-binding protein (IRBP): Ascension number AB082979). Target sequences are amplified using realtime qPCR in the presence of either a fluorescent nonspecific intercalating dye (used here) or a sequence specific fluorescent reporter molecule. Relative telomere length is calculated as the ratio of fluorescence from the telomeric amplicon compared to that from the non-variable copy gene standardised to a common sample ('gold sample') run on all plates (see equations below).

Primers

During assay development seven primer pairs targeting four control genes were designed from *Meles meles* sequences available in the GenBank database: Inter-photoreceptor retinoid-binding protein (IRBP), Actin alpha cardiac muscle protein (ACTC), Transthyretin

protein (TTR) and cystic fibrosis transmembrane conductance regulator (CFTR). Primer pairs which showed non-specific amplification or primer-dimer formation were discarded. A primer pair targeting the IRBP region was chosen (see Table 1.) owing to its superior performance and previous use in telomere assays (Izzo *et al.* 2011) and phylogenetic work (Sato *et al.* 2003) (owing to it being non-variable in copy number).

Table 1. Details the primer and oligonucleotide sequences used in the qPCR experiment.

Name	Target	Sequence
Tel1b	Telomeric Region	5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
Tel2b	Telomeric Region	5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'
IRBP-F	Inter-photoreceptor retinoid-binding protein (Ascension# AB082979)	5'-GCCACATTTCTGGTATCCCCT-3'
IRBP-R	Inter-photoreceptor retinoid-binding protein (Ascension# AB082979)	5'-GGGCGGTCGTAGATGGTATC-3'
Oligo-IRBP	NA	GCCACATTTCTGGTATCCCCTACTTCATCTCCTACCTGCACC CAGGGAACACAGTCCTGCACGTGGATACCATCTACGACCG CCC
Oligo-Telo	NA	$(TTAGGG)_{14}$

Plate setup

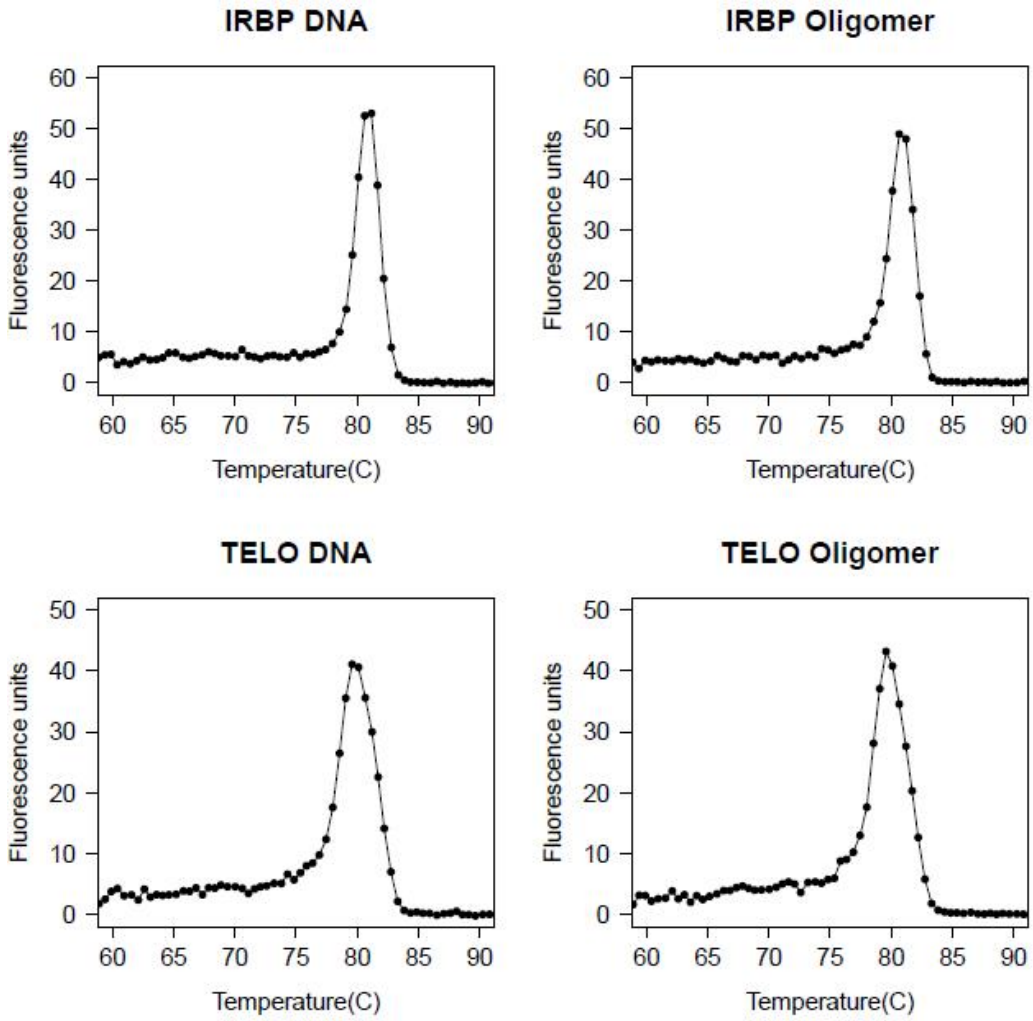
High-purity salt-free primers were synthesised by Eurofins (see Table 1.), diluted and stored at -20°C until use. Non-skirted 96-well Polypropylene qPCR plates were loaded manually and sealed with Mx3000P/Mx3005P Optical Strip Caps (Agilent). Control gene (IRBP) and telomere reactions were run on separate plates owing to differing optimal reaction temperatures (data not shown). Telomere and IRBP primer concentrations were optimised to 400nM and 200nM respectively. All reactions were run in triplicate (technical replicates) and averaged prior to analysis. In order to account for differences in amplification efficiencies between different plates, a standard curve was run on all plates comprising 1:2 serial dilutions of a pool of DNA from 10 individuals of unknown age (resulting in total DNA concentrations of 20, 10, 5, 2.5 and 1.25ng). In order to calculate a relative telomere length value for all samples, the 5ng dilution of the standard curve was used as the 'golden sample' to which all other samples were compared. See Table 2 for a schematic representation of the plate setup.

Table S2. Shows a schematic of the standardised plate qPCR plate set-up. All numbers refer to the replicate of an individual sample, NTC = No Template Control, GS = Gold Sample, numbers preceded by S denote standard curve dilutions whereby: S1 = 20ng, S2 = 10ng, S3 = 5ng, S4 = 2.5ng, and S5 = 1.25ng. For the absolute qPCR telomere length estimation and inter-plate variation experiment, samples 3-7 were replaced by one in ten dilutions of known concentrations of synthesised oligomers (see table 1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	3	3	3	11	11	11	19	19	19
B	S2	S2	S2	4	4	4	12	12	12	20	20	20
C	S3/GS	S3/GS	S3/GS	5	5	5	13	13	13	21	21	21
D	S4	S4	S4	6	6	6	14	14	14	22	22	22
E	S5	S5	S5	7	7	7	15	15	15	23	23	23
F	1	1	1	8	8	8	16	16	16	24	24	24
G	2	2	2	9	9	9	17	17	17	25	25	25
H	NTC	NTC	NTC	10	10	10	18	18	18	26	26	26

Individuals were randomly allocated to qPCR plates (all samples from a given individual were run on the same plate in order remove the impact of inter-plate variation on within-individual telomere length comparisons). The final reaction volume was 20ul containing 10ul of Brilliant II SYBR® Green Low ROX QPCR Master Mix (Agilent), 4ul nuclease free water (Fisher), 1ul each of forward and reverse primers (see Table 1) and 4ul of 1.25ng/ul DNA sample (or 4ul nuclease free water for the no template control). Reactions were run on the Stratagene Mx3000P qPCR system using a two-step reaction profile (Control Gene: 10 mins at 95°C, followed by 40 cycles of 30s at 95°C and 1min at 60°C, Telomere: 10 mins at 95°C, followed by 40 cycles of 30s at 95°C and 1min at 56°C). Fluorescence was recorded at the end of the low temperature annealing/extension step. LinRegPCR (v2013.0) was used to correct for baseline fluorescence and determine the window of linearity per amplicon. The threshold values (Nq) were set at the centre of the window in linearity for each amplicon (Nq = 0.22 and 0.17 (log fluorescence units) for IRBP and telomere reactions respectively). Threshold cycle values (Cq) for each sample were then determined as the cycle at which the amplification plot crossed the Nq. Primer specificity was confirmed through melt curve analysis (see Figure 1) and observation of a single band of the expected size after electrophoresis on a 3% agarose gel.

Figure 1. Shows the melt curve analysis of both DNA and oligomer templates.



Calculations

Cq values were plotted against log concentration in order to determine the amplification efficiency of both IRBP and Telomere primers for each plate pair run. Across all plates, the mean standard curve amplification efficiencies were 99.9% (SE ± 1.5) for IRBP primers and 99.2% (SE ± 0.9) for the telomere primers. The R^2 for each standard curve was >0.99.

First, two initial starting quantities (X_0) were calculated for each sample, one from its telomere plate (X_0 TEL) and one from its IRBP plate (X_0 IRBP). To reconcile for amplification efficiency differences between plate runs we used the following equation:

$$X_0 = 10^{(Cq-b)/m}$$

Where Cq = Cycle at which the focal sample crosses the threshold (Nq), b = plate specific intercept of the log of the standard curve and m = plate specific slope of the log of the standard curve.

The amount of telomere in the focal sample was then normalised to the initial quantity of DNA in the sample by calculating:

$$X_0 \text{ sample} = X_0 \text{ TEL} / X_0 \text{ IRBP}$$

Finally, relative telomere length (RTL) was calculated by normalising the focal sample to the golden sample:

$$\text{RTL} = X_0 \text{ sample} / X_0 \text{ golden sample}$$

Repeatability

Amplicon specific within-plate variability was determined by examining the standard deviation of the triplicate Cq values for each sample across each plate. The median and inter-quartile range of the standard deviations across all samples (n=361) was 0.054 (0.036-0.082) for the IRBP primers and 0.097 (0.059- 0.14) for the telomere primers. In order to determine between-plate repeatability, 21 randomly selected samples (a single plate) were each run three times (each run once for telomere and once for IRBP, totalling 6 plates). The coefficient of variation in the relative telomere length estimates across all samples was 7.5%.

Absolute Telomere Length Estimation

The 21 samples selected for between-plate repeatability analysis (see above) were also subjected to the absolute telomere estimation method described by O'Callaghan & Fenech (2011). This method allows the calibration of relative telomere length estimates obtained using qPCR to known quantities of synthetic telomere and control gene oligomers (see Table 1 for sequences used here). In addition to the standard curve required for the relative method (see above) we included ten-fold dilutions of known concentrations of each synthetic oligomer on the same plate. Determining where each DNA sample crosses the synthetic standard curve for each amplicon can be used in order to determine the absolute

quantity of telomere in each sample. Melt-curve analysis showed that primer products from both DNA and oligomer templates were specific (single peak) and had the same melt temperatures (Figure 1). As synthetic oligomers may have different amplification efficiencies to biologically extracted DNA (leading to bias in absolute telomere quantities), we calculated absolute starting quantities for each amplicon (A_0: kb for telomere amplicon and diploid genomes for IRBP amplicon) whilst reconciling for differences in amplification efficiency between synthetic oligomers and extracted DNA samples as follows:

$$A_0 = E_{DNA}{}^{b_{Oligo} log E_{DNA}(E_{Oligo}) - Cq_{sample}}$$

Where DNA = Biological extracted DNA, Oligo = synthetic oligomer, Cq = Cycle at which the focal sample crosses the threshold (Nq), b = intercept of the log of the standard curve, E = Efficiency of standard curve $10^{(1/-m)}$ and m = slope of the log of the standard curve.
We then standardised the absolute amount of telomere to the number of diploid genomes contained in the sample as follows:

$$\text{Absolute TL} = A_0\,\text{TEL} \,/\, A_0\,\text{IRBP}$$

Relative to Absolute Conversion

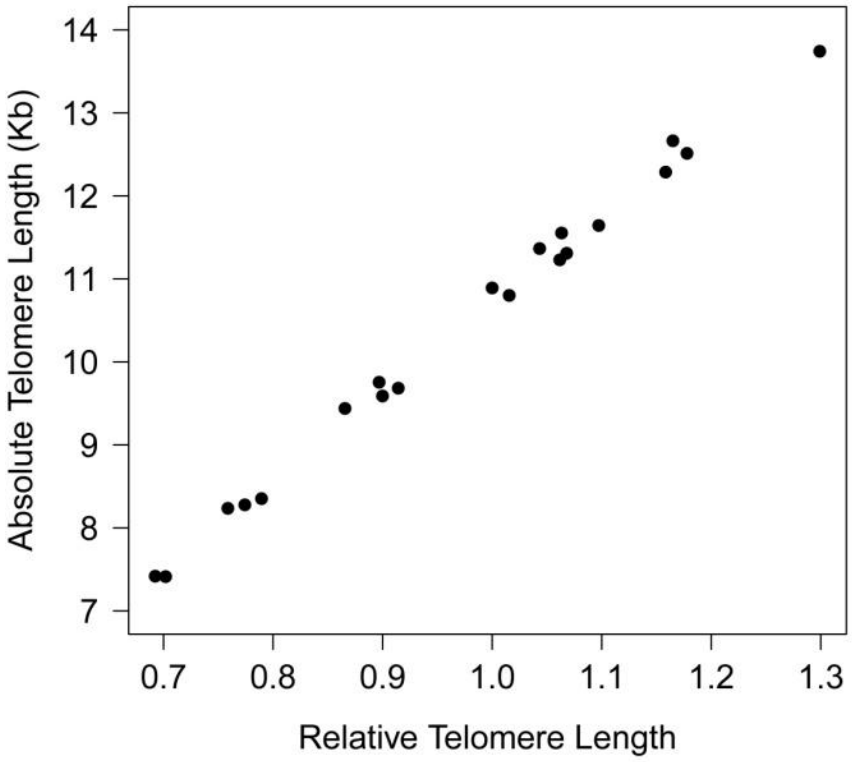
The estimates derived from the relative and absolute methods for these 21 samples were linearly related and highly correlated (R^2 >0.99, Figure 2). Given that badgers have 22 chromosome pairs (44 chromosomes = 88 telomeres) and that the IRBP is a single copy gene (Sato *et al.* 2003), it was possible define an equation for the conversion of the relative telomere lengths into absolute telomere length per chromosome end:

$$\text{Absolute TL Estimate (Kb)} = (8.5 + 932.9*(\text{RTL Estimate}))/88$$

We estimated the average immune cell telomere length in our population of European badgers to be ~10kb, which is similar to immune cell telomere length estimates in humans (~15kb in young individuals; Vera *et al.* 2012). It is important to note that the average immune cell telomere length estimate quoted here must be treated with caution. This

estimate has not been validated a secondary direct methodology (such as TRF) (Foote *et al.* 2013; Nussey *et al.* 2014).

Figure 2. shows the correlation between absolute telomere length estimates and relative telomere length estimates for the 21 randomly selected samples.



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Appendix B – Full model selection output (Chapter 2)

Table 1. Full model selection output for the analysis in Chapter 2. Where; ✓ = denotes terms factors included in the model; INT = Intercept; MA = Mean Age (Years); DA = Δ Age (Years); TB = bTB status; SMI = body condition (Scaled Mass Index); df = Degress of freedom; LogLik = Log Liklihood; AICc = Akaike's Information criterion corrected for small sample size; W = Model weight; grey shading = models included in the 'top set'.

INT	MA	DA	MA^2	DA^2	DA:MA	SEX	SMI	TB	DA:SEX	DA:SMI	DA:TB	MA:SEX	MA:SMI	MA:TB	SEX:SMI	SEX:TB	df	logLik	AICc	ΔAICc	W	AW
10.01	-0.14	-0.47						✓									9	-615	1249.3	0.00	0.19	0.7
10.05	-0.13	-0.45		-0.60				✓									10	-615	1249.9	0.67	0.13	0.0
10.13	-0.13	-0.46															7	-618	1251.0	1.70	0.08	0.3
10.00	-0.13	-0.47	0.00					✓									10	-615	1251.4	2.11	0.07	0.0
10.17	-0.12	-0.44		-0.60													8	-618	1251.6	2.31	0.06	0.0
10.02	-0.09	-0.45	-0.01	-0.63				✓									11	-615	1252.0	2.69	0.05	0.0
10.13	-0.13	-0.69			0.07												8	-618	1252.3	3.00	0.04	0.0
10.06	-0.13	-0.46				✓											8	-618	1252.4	3.15	0.04	0.0
10.13	-0.13	-0.48					0.04										8	-618	1252.8	3.58	0.03	0.0
10.09	-0.12	-0.44		-0.65		✓											9	-617	1252.9	3.58	0.03	0.0
10.06	-0.13	-0.72				✓			✓								9	-617	1252.9	3.68	0.03	0.0
10.09	-0.09	-0.46	0.00														8	-618	1253.0	3.70	0.03	0.0
9.97	-0.11	-0.47						✓						✓			11	-615	1253.3	4.05	0.03	0.0
10.01	-0.14	-0.55						✓			✓						11	-615	1253.3	4.07	0.02	0.0
10.17	-0.12	-0.47		-0.62			0.05										9	-617	1253.4	4.14	0.02	0.0
10.11	-0.06	-0.44	-0.01	-0.65													9	-617	1253.4	4.16	0.02	0.0
9.92	-0.14	-0.46				✓		✓								✓	12	-614	1253.6	4.34	0.02	0.0
10.13	-0.13	-0.50					0.03			0.17							9	-618	1254.2	4.92	0.02	0.0
10.02	-0.11	-0.46				✓						✓					9	-618	1254.3	5.04	0.02	0.0
10.03	-0.09	-0.46	0.00			✓											9	-618	1254.4	5.17	0.01	0.0
10.13	-0.13	-0.49					-0.03						0.02				9	-618	1254.5	5.23	0.01	0.0
10.03	-0.06	-0.44	-0.01	-0.69		✓											10	-617	1254.7	5.42	0.01	0.0
10.09	-0.09	-0.48	-0.01				0.04										9	-618	1254.8	5.57	0.01	0.0
10.11	-0.05	-0.47	-0.01	-0.67			0.05										10	-617	1255.2	5.93	0.01	0.0
10.06	-0.13	-0.51				✓	-0.06								✓		10	-617	1255.5	6.21	0.01	0.0
10.12	-0.12																6	-622	1256.0	6.74	0.01	0.0
9.79		-0.45															6	-623	1258.6	9.34	0.00	0.0
9.80																	5	-627	1263.4	14.09	0.00	0.0

Figure 1. Patterns of body length change with age. (A) illustrates that the rate of change in body length decreases with increasing age. Failure to account for this would result in biased estimates of growth rate depending on the age at which individuals were captured. (B) illustrates that taking the $\log_{10}$(age) reduced this source of bias and thus made comparisons of growth rates taken at different ages possible. Points present data points and black lines present the best supported model relating age to length in each case (from a choice of either linear of quadratic). We only estimate growth rates using body length data from individuals of up to and including 300 days (as opposed to 365 days: red points after the dashed line in Figure B) because growth slows dramatically during the winter months, violating the assumption of log-linearity. No data exists for individuals less than 75 days of age as welfare regulations do not permit the trapping of individuals still dependent on lactation.

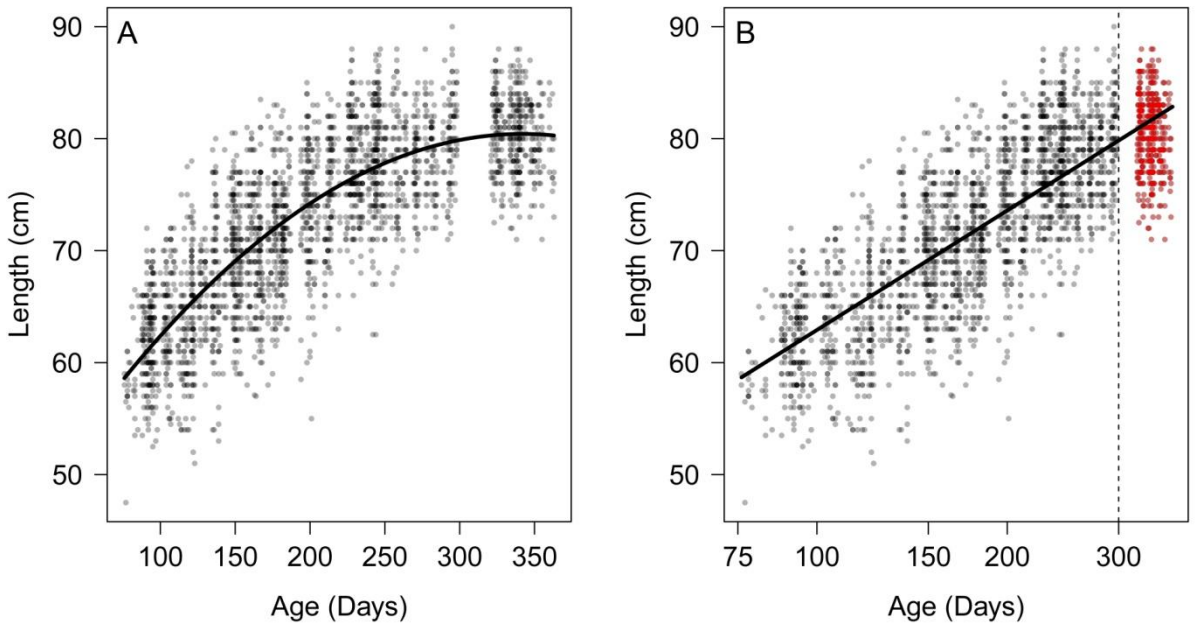
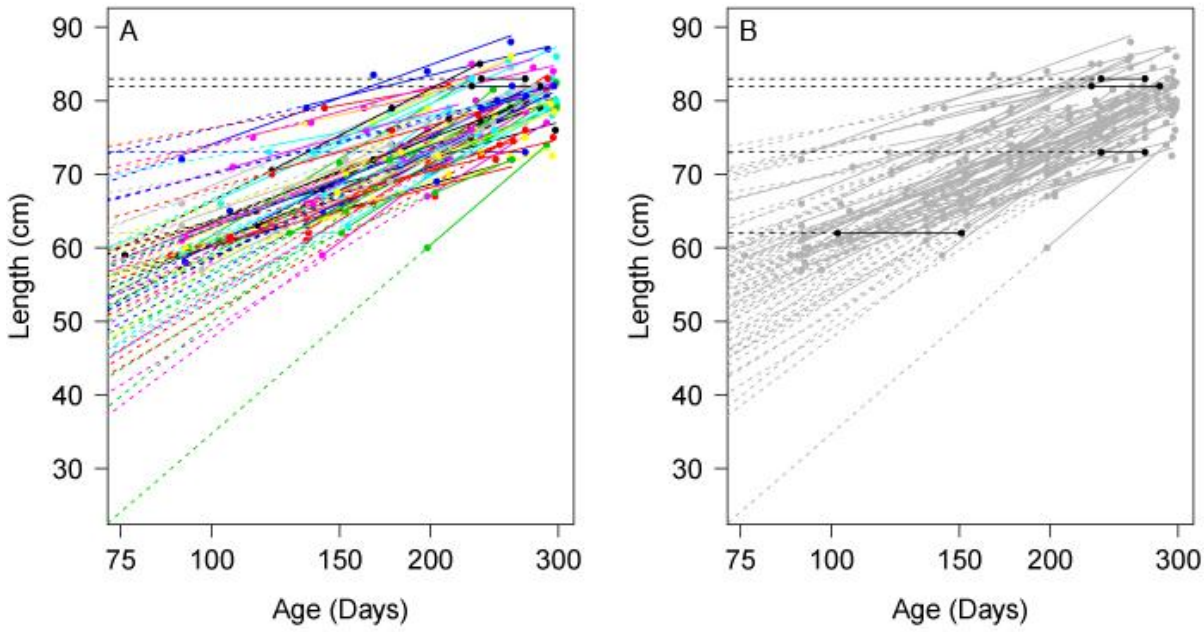


Figure 1. Calculation of individual growth rates and outlier exclusion. (A) shows the calculation of growth rates for each individual badger in the dataset where; circles denote length measurements, gradient of the solid line is the growth rate and dashed lines represent the extrapolated gradients in order to determine the 'growth intercepts'. (B) shows the exclusion of four outliers with zero or negative growth rates (black points and lines).



Appendix E – Full model selection output (Chapter 3)

Table 1. Unabridged output from model selection in Chapter 3. Where: ✓= factors included in the model; IN = Intercept, ΔA = Delta Age (Years), MA = Mean Age (Years), S = Sex, TB = bTB Status, GI = Growth Intercept, GR = Growth Rate, AL = Adult Length, AR = August rainfall, MR = May rainfall, D = Density, : = an interaction between two parameters, df = Degrees of freedom, ΔAICc = change in Akaike Information Criterion corrected for small sample size from the best supported model, AW = adjusted model weight after removal of models ΔAICc>6 from the best supported model and exclusion any models of which a simpler nested version attained stronger support, grey shaded area = models included in the 'top set'.

IN	ΔA	MA	TB	GI	GR	ΔA:GR	S	GR:S	GR:TB	AL	AL:GR	AR	MR	GR:AR	GR:MA	ΔA:AR	ΔA:MR	AR:S	MR:S	AL:AR	AL:MR	D	D:RA	D:ΔA	D:S	D:AL	D:AR	D:MR	df	ΔAICc	AW
9.8	-0.49	-0.11	+									0.38																	10	0.00	0.89
9.9	-0.49	-0.12	+							-0.18		0.38																	11	1.05	-
9.6	-0.49	-0.11	+				✓					0.55						✓											12	1.63	-
9.9	-0.49	-0.13	+		-0.11							0.38																	11	1.84	-
9.8	-0.51	-0.11	+									0.37				-0.13													11	2.03	-
9.8	-0.49	-0.11	+									0.38	0.00																11	2.27	-
9.9	-0.49	-0.12	+							-0.18		0.38								0.01									12	3.36	-
9.9	-0.50	-0.13	+		-0.10							0.42		0.14															12	3.66	-
9.9	-0.49	-0.13	+	0.02	-0.09							0.38																	12	4.14	-
10.1	-0.49	-0.13	+																										9	4.22	0.11
9.9	-0.49	-0.11	+										-0.10									-0.12						0.33	12	4.26	-
9.8	-0.49	-0.11	+									0.38										0.02					0.07		12	4.37	-
9.9	-0.52	-0.10	+										-0.10				0.39												11	4.86	-
10.1	-0.49	-0.14	+							-0.16																			10	5.53	-
10.0	-0.49	-0.14	+				✓																						10	5.86	-
9.9	-0.50	-0.13	+	0.03	-0.07							0.42		0.14															13	5.99	-
10.1	-0.49	-0.14	+	0.11																									10	6.08	-
10.1	-0.49	-0.14	+		-0.10																								10	6.11	-
9.9	-0.49	-0.10	+										-0.10																10	6.15	-
10.1	-0.49	-0.13	+																			-0.02							10	6.46	-
10.1	-0.48	-0.16	+							-0.28												-0.05				-0.31			12	6.51	-
10.2	-0.49	-0.14	+		-0.04								0.01		0.36														12	6.68	-
9.9	-0.49	-0.08	+							-0.21			-0.19																11	6.88	-
10.1	-0.49	-0.15	+		-0.10	-0.24																							11	7.12	-
10.1	-0.49	-0.14	+							-0.17												-0.05							11	7.73	-
10.0	-0.49	-0.11	+		-0.13								-0.13																11	7.91	-
10.1	-0.49	-0.16	+		-0.08																	-0.02	-0.25						12	7.93	-
10.0	-0.48	-0.12	+		0.04				✓																				12	8.09	-
10.1	-0.49	-0.14	+	0.31	0.20																								11	8.34	-
10.1	-0.49	-0.15	+		-0.10																	-0.02							11	8.37	-
10.0	-0.47	-0.13	+																			-0.02		0.08					11	8.63	-
9.9	-0.49	-0.08	+							-0.22			-0.17								0.08								12	8.83	-
10.2	-0.49	-0.13	+	-0.18	-0.22								0.00		0.36														13	9.00	-
10.1	-0.49	-0.15	+		-0.09					-0.18	-0.14																		12	9.29	-
10.0	-0.49	-0.14	+	0.36	0.24	-0.24																							12	9.37	-
10.0	-0.49	-0.16	+		-0.25		✓	✓																					12	9.50	-
9.9	-0.49	-0.11	+				✓						-0.04						✓										12	10.08	-
10.0	-0.49	-0.15	+	2.58	2.43					-0.38	-0.14																		13	10.11	-
10.1	-0.49	-0.15	+	0.40	0.30																	-0.01	-0.26						13	10.20	-
10.0	-0.49	-0.11	+	-0.08	-0.21								-0.13																12	10.21	-
10.0	-0.49	-0.14	+				✓															0.00			✓				12	10.35	-
10.0	-0.48	-0.12	+	0.12	0.16				✓																				13	10.41	-
10.1	-0.49	-0.14	+	0.28	0.17																	-0.01							12	10.64	-
9.9	-0.50	-0.15	+	0.82	0.52		✓	✓																					13	11.56	-

Figure 1. The relationship between the growth intercept (proxy for early life size or birth date), both growth rate and final adult size. The points represent the raw data (one point per individual) the dashed lines represent the linear regression between growth axis and growth rate (A) and growth intercept and final adult size (B). Both gradients differ significantly from zero at the 99% level.

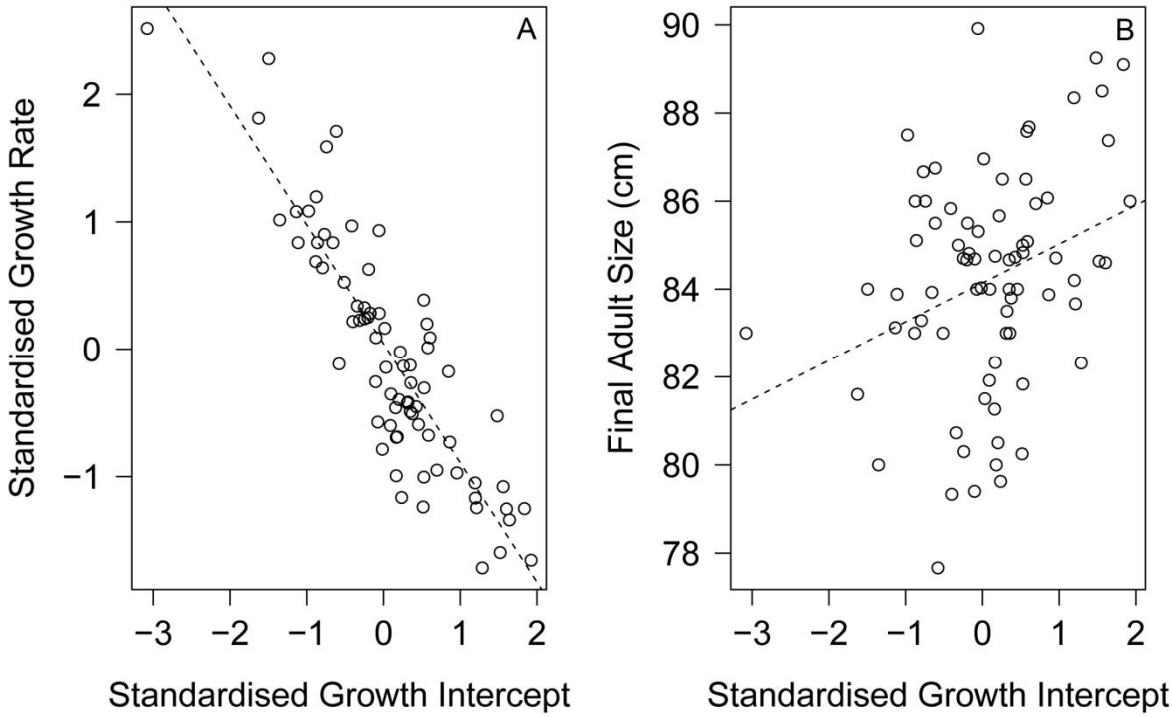


Table 1. The full unabridged model selection table output from Chapter 4. Where: Int = Intercept, bTB = Current bovine tuberculosis status, Cond = Condition (scaled mass index), df = Degrees of freedom, AICc = Akaike's Information Criterion corrected for small sample size, ΔAICc = deviation in AICc from the best supported model, W = Model weight, AW = Adjusted model weight after exclusion of models with ΔAICc<6 from the top model and models with a simpler, nested version with more model weight (support), ✓ = terms included in the model, : = interactions.

Int	Δ Age	Mean Age	Sex	bTB	Cond	Δ Age * Mean Age	Δ Age * Sex	Δ Age *bTB	df	logLik	AICc	ΔAICc	W	AW
0.32	-0.03	-0.02	✓	✓					10	736.9	-1453.50	0.00	0.23	0.81
0.36	-0.03	-0.02	✓	✓	-0.01				11	737.8	-1453.40	0.14	0.21	0.00
0.32	-0.04	-0.02	✓	✓		0.00			11	737.0	-1451.70	1.79	0.09	0.00
0.36	-0.04	-0.02	✓	✓	-0.01	0.00			12	738.0	-1451.60	1.89	0.09	0.00
0.32	-0.04	-0.02	✓						8	733.4	-1450.70	2.84	0.06	0.20
0.36	-0.03	-0.02		✓	-0.01				10	735.2	-1450.20	3.34	0.04	0.00
0.35	-0.04	-0.02	✓		0.00				9	734.1	-1450.00	3.48	0.04	0.00
0.31	-0.03	-0.02		✓					9	733.9	-1449.60	3.96	0.03	0.00
0.32	-0.03	-0.02	✓	✓			✓	✓	13	737.9	-1449.30	4.19	0.03	0.00
0.36	-0.03	-0.02	✓	✓	-0.01		✓	✓	14	738.8	-1449.20	4.33	0.03	0.00
0.32	-0.04	-0.02	✓			0.00			9	733.6	-1449.10	4.42	0.03	0.00
0.32	-0.03	-0.02	✓				✓		9	733.4	-1448.70	4.83	0.02	0.00
0.35	-0.04	-0.02	✓		0.00	0.00			10	734.4	-1448.50	4.99	0.02	0.00
0.36	-0.04	-0.02		✓	-0.01	0.00			11	735.4	-1448.50	5.07	0.02	0.00
0.35	-0.03	-0.02	✓		0.00		✓		10	734.1	-1448.00	5.51	0.01	0.00
0.36	-0.03	-0.02		✓	-0.01			✓	12	736.1	-1447.90	5.57	0.01	0.00
0.31	-0.04	-0.02		✓		0.00			10	734.0	-1447.80	5.74	0.01	0.00
0.31	-0.03	-0.02		✓				✓	11	734.8	-1447.30	6.18	0.01	0.00
0.35	-0.04	-0.02			-0.01				8	731.1	-1446.10	7.46	0.01	0.00
0.31	-0.04	-0.02							7	730.1	-1446.00	7.50	0.01	0.00
0.31	-0.04	-0.02							7	730.1	-1446.00	7.50	0.01	0.00
0.31	-0.04	-0.02				0.00			8	730.3	-1444.50	9.03	0.00	0.00
0.34			✓	✓	-0.01				9	704.8	-1391.50	62.01	0.00	0.00
0.34				✓	-0.01				8	703.5	-1390.80	62.69	0.00	0.00
0.29			✓	✓					8	703.3	-1390.40	63.08	0.00	0.00
0.28				✓					7	701.6	-1389.10	64.40	0.00	0.00
0.31			✓		-0.01				7	693.2	-1372.40	81.16	0.00	0.00
0.27			✓						6	692.2	-1372.30	81.22	0.00	0.00
0.31					-0.01				6	691.5	-1370.80	82.67	0.00	0.00
0.26									5	690.1	-1370.20	83.31	0.00	0.00

Table 1. Removal of infected individuals. The model selection table output after exclusion of all known infected individuals (those classed as 'exposed' or 'excretor'). Where: Int = Intercept, Cond = Condition (scaled mass index), df = Degrees of freedom, AICc = Akaike's Information Criterion corrected for small sample size, ΔAICc = deviation in AICc from the best supported model, W = Model weight, AW = Adjusted model weight after exclusion of models with ΔAICc<6 from the top model and models with a simpler, nested version with more model weight (support), ✓ = terms included in the model, : = interactions.

Int	Δ Age	Mean Age	Sex	Cond	Δ Age * Mean Age	Δ Age * Sex	df	logLik	AICc	ΔAICc	W	AW
0.32	-0.04	-0.02	✓				8	733.4	-1450.7	0.00	0.29	0.84
0.35	-0.04	-0.02	✓	0.00			9	734.1	-1450.0	0.64	0.21	0.00
0.32	-0.04	-0.02	✓		0.00		9	733.6	-1449.1	1.58	0.13	0.00
0.32	-0.03	-0.02	✓			✓	9	733.4	-1448.7	1.99	0.11	0.00
0.35	-0.04	-0.02	✓	0.00	0.00		10	734.4	-1448.5	2.15	0.10	0.00
0.35	-0.03	-0.02	✓	0.00		✓	10	734.1	-1448.0	2.67	0.08	0.00
0.35	-0.04	-0.02		-0.01			8	731.1	-1446.1	4.62	0.03	0.08
0.31	-0.04	-0.02					7	730.1	-1446.0	4.65	0.03	0.08
0.31	-0.04	-0.02			0.00		8	730.3	-1444.5	6.19	0.01	0.00
0.31			✓	-0.01			7	693.2	-1372.4	78.32	0.00	0.00
0.27			✓				6	692.2	-1372.3	78.37	0.00	0.00
0.31				-0.01			6	691.5	-1370.8	79.83	0.00	0.00
0.26							5	690.1	-1370.2	80.47	0.00	0.00

Appendix I – Unabridged multivariate model output (Chapter 4)

Appendix I shows the unabridged model output from the multivariate modelling process for random effects, fixed effects and the within-and between-individual posterior correlations for the two traits. CI = 'Credibility Interval'

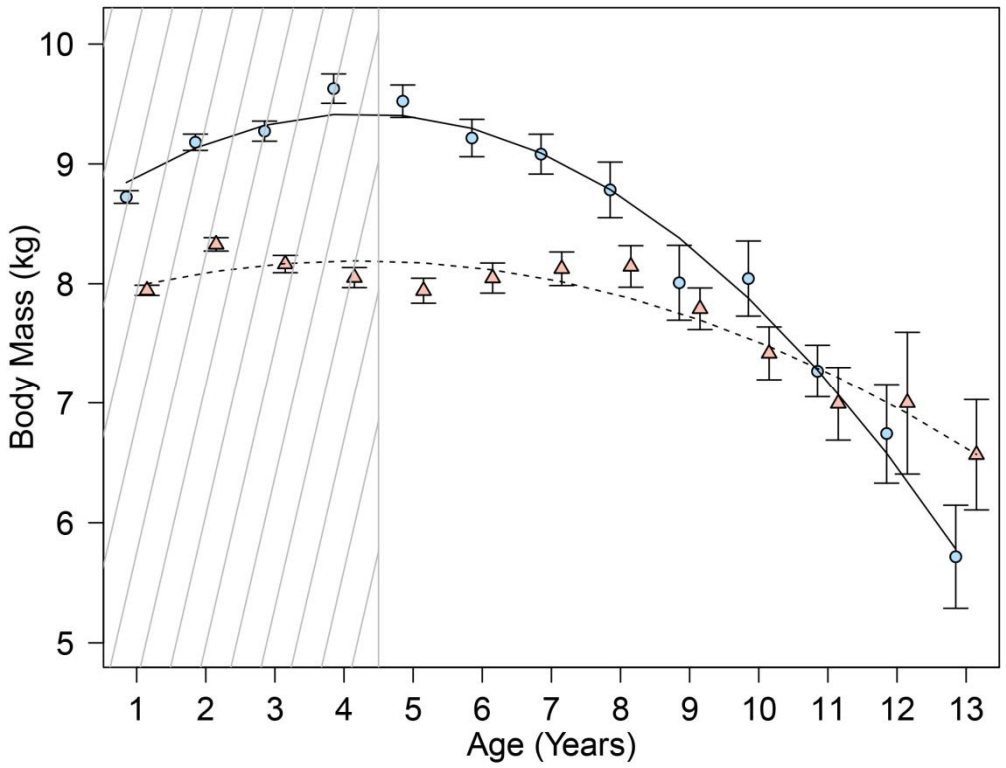
Random Effects			
G-structure	Mean	Lower 95% CI	Upper 95% CI
Telomere Variance	0.792	0.491	1.169
Telomere/IFNγ Covariance	0.000	-0.016	0.018
IFNγ Variance	0.011	0.009	0.013
Plate (Telomere)	0.789	0.227	1.561
Plate (IFN)	0.005	0.003	0.006
R-structure:	Mean	Lower 95% CI	Upper 95% CI
Telomere Variance	1.171	0.940	1.387
Telomere/IFNγ Covariance	-0.001	-0.013	0.012
IFNγ Variance	0.009	0.008	0.010
Fixed Effects	Mean	Lower 95% CI	Upper 95% CI
Intercept (Telomere)	10.007	9.494	10.588
Intercept (IFNγ)	0.294	0.265	0.325
Mean Age (Telomere)	-0.067	-0.165	0.046
Mean Age (IFNγ)	-0.016	-0.024	-0.009
Δ Age (Telomere)	-0.480	-0.755	-0.222
Δ Age (IFNγ)	-0.033	-0.047	-0.021
Sex (IFNγ: Female)	0.029	0.000	0.055
bTB (Telomere: Exposed)	0.431	0.064	0.814
bTB(Telomere: Excretor)	-0.161	-0.875	0.634
bTB(IFNγ: Exposed)	-0.008	-0.030	0.013
bTB(IFNγ: Exposed)	-0.053	-0.104	0.000
Posterior Correlation	Mean	Lower 95% CI	Upper 95% CI
Within: Telomere/IFNγ	-0.007	-0.132	0.119
Between: Telomere/IFNγ	-0.029	-0.182	0.184

Appendix J – Full model selection output (Chapter 5)

Table 1. Model selection table on the factors affecting body mass during the senescent period. The abridged version is presented as Table 1 in Chapter 5 of the main manuscript. The final column indicates those models that were retained after the application of the nesting rule (Richards *et al.* 2011; only these models appear in Chapter 5:Table 1 in the main manuscript), whereby models are removed if they are more complex versions of nested (simpler) models that attracted stronger support. The grey area denotes the models included in the top set (the second model in the table was excluded following the nesting rule), ✓ = terms included in the model, * = interaction between two terms, Int = intercept, SGS = Social Group Size, ALC = Age at Last Capture, Age = Age in days, Age_{cat} = Age coded categorically (years), LYC = Last Year of Capture, df = degrees of freedom, AICc = Akaike's Information Criterion corrected for small sample size, ΔAICc = change in AICc relative to best supported model, AW = adjusted weight after removal of more complex models with less support.

Int	Sex	Month	SGS	ALC	Age	Age^2	Age*Sex	Age^2*Sex	LYC	Sex*LYC	Age_{cat}	Age_{cat}*Sex	df	logLik	ΔAICc	AW	RET
8.67	✓	✓	-0.02	-0.04	0.42	-0.03	✓		✓				23	-1791	0.00	0.89	✓
8.67	✓	✓	-0.02	-0.04	0.42	-0.03	✓		✓	✓			24	-1791	1.65	0.00	
8.76	✓	✓	-0.02	-0.04	0.39	-0.03	✓	✓	✓				24	-1791	2.01	0.00	
8.76	✓	✓	-0.02	-0.04	0.39	-0.03	✓	✓	✓	✓			25	-1791	3.65	0.00	
8.43	✓	✓	-0.02	0.00	0.39	-0.03	✓						22	-1794	4.25	0.11	✓
8.48	✓	✓	-0.02	0.00	0.38	-0.03	✓	✓					23	-1794	6.30	0.00	
9.00	✓	✓	-0.02	-0.04	0.35	-0.03			✓				22	-1800	15.89	0.00	
8.97	✓	✓	-0.02	-0.04	0.35	-0.03			✓	✓			23	-1799	16.60	0.00	
8.76	✓	✓	-0.02	0.00	0.32	-0.03							21	-1804	20.62	0.00	
9.98	✓	✓	-0.02	-0.04					✓		✓	✓	36	-1788	20.71	0.00	
9.99	✓	✓	-0.02	-0.04					✓	✓	✓	✓	37	-1788	22.71	0.00	
9.98	✓	✓	-0.02	-0.04					✓		✓		28	-1798	23.79	0.00	
9.62	✓	✓	-0.02	0.00							✓	✓	35	-1791	23.84	0.00	
9.96	✓	✓	-0.02	-0.03					✓	✓	✓		29	-1797	24.65	0.00	
9.60	✓	✓	-0.02	0.00							✓		27	-1801	27.21	0.00	
10.72	✓	✓	-0.02	-0.03	-0.12				✓				21	-1808	30.06	0.00	
10.72	✓	✓	-0.02	-0.03	-0.12				✓	✓			22	-1808	31.27	0.00	
10.49	✓	✓	-0.02	0.01	-0.15								20	-1812	34.90	0.00	
10.68	✓	✓	-0.02	-0.12					✓				20	-1824	58.50	0.00	
10.68	✓	✓	-0.02	-0.12					✓	✓			21	-1823	60.29	0.00	
9.97	✓	✓	-0.02	-0.07									19	-1844	97.08	0.00	

Figure 1. Removal of individuals prior to peak body mass. Observations prior to five years of age (grey hatched area) where removed from all body mass analyses to ensure that the age-related body mass dynamics prior to the attainment of peak body mass (which likely relate more to growth than senescence) did not influence our statistical conclusions regarding the age-related body mass dynamics in later life. The mean body masses and standard errors for males (blue circles) and females (red triangles) for each year of age, for all individuals aged one year and over. The lines represent quadratic regression lines fitted to the means for males (solid line) and females (dashed line). The hatched grey area represents the age-classes excluded from the senescence analysis dataset.



In order to address the possibility that males show steeper late-life declines in body mass simply because they are larger individuals with more body mass to lose, we repeated our analysis from Section A in the main paper using a metric of standardised body condition, the Scaled Mass Index (SMI; Peig & Green 2010) in place of body mass. The SMI accounts for the sexual dimorphism in body size by scaling the body masses of all individuals (of differing body sizes) to the value expected for a single standardised body length, utilising the species-specific allometric scaling relationship between body length and body mass (Peig & Green 2010). Accordingly, the sex difference in mean body mass in European badgers in our data set (Males: 8.85kg, Females: 7.77kg) is no longer apparent in SMI (Males: 8.74kg, Females: 8.67kg) and, likewise, the higher variance in body mass among males due most likely to their larger size (Males: 2.95, Females: 2.55) is no longer apparent in SMI, indeed the reverse is true (Males: 2.04, Females: 2.72). Using SMI, body mass losses are therefore weighted against an individual's size, leaving a larger absolute change in body mass required in longer individuals than shorter individuals to bring about a comparable change in SMI. The SMI has been found to capture variation in fat and protein reserves more effectively than traditional residual body condition indices (Peig & Green 2009), and thus makes it an appropriate index for use in senescence studies. Body length measurements (the distance in centimetres from the tip of the nose to the distal point of the last caudal vertebra) were only available for badger captures from 1997 onwards, which substantially reduced the data set of captures of known-age individuals in late-life to just 536 observations (a reduction of 57%) when using SMI as the response variable in place of body mass. The 'scaling component' was estimated through standardised major axis regression of ln(body mass) on ln(body length) to be 4.5 (following Peig & Green 2010).

Repeating the analysis in Section A of the main manuscript using SMI as the response term in place of body mass confirmed support for a sex difference in the rate of standardised body condition (SMI) loss with age in late-life, whereby the SMIs of males declined at a faster rate than those of females (Figure S2a), again while controlling for terminal effects, selective disappearance effects, current social group size, month of capture and the random effects (Table S2). Consistent with the results of the body mass modelling presented in the

main text, the SMI analysis suggests that SMI decreases with increasing age at last capture (a selective disappearance effect; Figure S2b) and in the last year of capture (a terminal effect; Figure S2c). Whilst the best-supported model contained the age*sex interaction, two models within the ΔAICc < 6 top model set did not (Table S2), which likely reflects the marked reduction in the available sample size when using SMI in place of body mass .

1.

Peig, J. & Green, A.J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. *Oikos*, 118, 1883–1891.

2.

Peig, J. & Green, A.J. (2010). The paradigm of body condition: a critical reappraisal of current methods based on mass and length. *Funct. Ecol.*, 24, 1323–1332.

3.

Richards, S., Whittingham, M. & Stephens, P. (2011). Model selection and model averaging in behavioural ecology: the utility of the IT-AIC framework. *Behav. Ecol. Sociobiol.*, 65, 77–89.

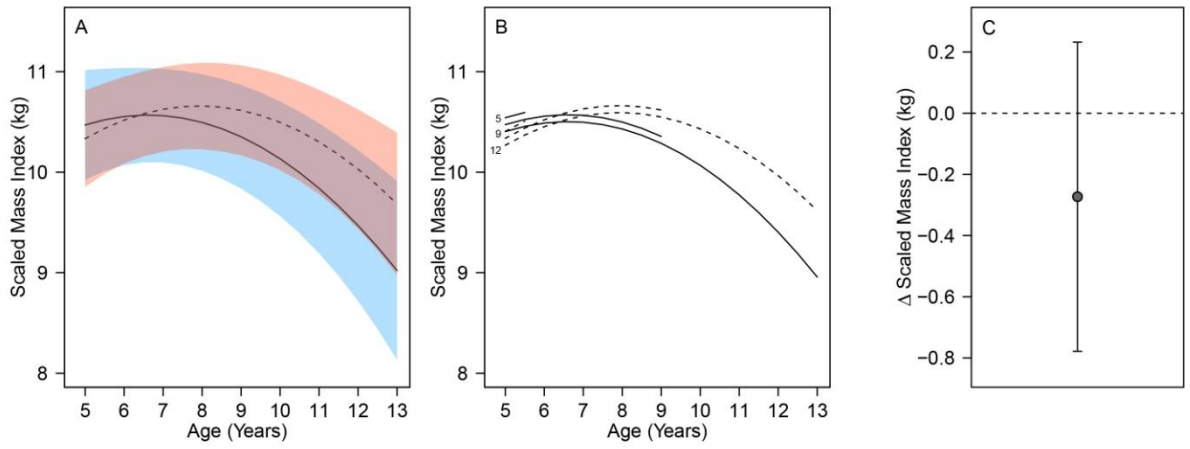


Figure 1. Model output from body condition analyses. (A) presents predicted SMI of males (blue/solid line) and females (red/dashed line) with advancing age from the top model in Table A2. Predictions were made for badgers outside of their year of last capture, with ALC and social group size set to their mean values (9.2 and 12 respectively), and month set to July. The shaded areas represent 95% confidence intervals based on fixed effects uncertainty. (B) presents the effect of age at last capture (ALC) for males (solid lines) and females (dashed lines) for individuals last caught at ages 5, 9 and 12 years. (C) presents the terminal effect; the predicted change in SMI of individuals in their last year of capture (whiskers present the 95% confidence interval).

Int	Sex	Month	SGS	ALC	Age	Age^2	Age*Sex	Age^2*Sex	LYC	Sex*LYC	df	ΔAICc	AW	RET
8.94	✓	✓	-0.08	-0.02	0.60	-0.04	✓		✓		21	0.00	0.55	✓
8.92	✓	✓	-0.08	-0.02	0.61	-0.04	✓		✓	✓	22	0.78	0.00	
9.15	✓	✓	-0.08	-0.03	0.58	-0.04			✓		20	1.19	0.30	✓
9.11	✓	✓	-0.08	-0.02	0.56	-0.04	✓	✓	✓		22	2.01	0.00	
9.12	✓	✓	-0.08	-0.02	0.55	-0.03	✓	✓	✓	✓	23	2.72	0.00	
9.17	✓	✓	-0.08	-0.03	0.58	-0.04			✓	✓	21	3.13	0.00	
8.48	✓	✓	-0.08	0.04	0.58	-0.04	✓				20	3.22	0.11	✓
8.66	✓	✓	-0.08	0.04	0.54	-0.04	✓	✓			21	5.21	0.00	
8.68	✓	✓	-0.08	0.04	0.56	-0.04					19	5.26	0.04	✓
11.74	✓	✓	-0.08	-0.08					✓		18	13.52	0.00	
11.71	✓	✓	-0.08	-0.03	-0.06				✓		19	13.71	0.00	
11.75	✓	✓	-0.08	-0.08					✓	✓	19	15.13	0.00	
11.71	✓	✓	-0.08	-0.04	-0.05				✓	✓	20	15.53	0.00	
11.32	✓	✓	-0.08	0.04	-0.12						18	19.09	0.00	
11.09	✓	✓	-0.07	-0.05							17	28.88	0.00	

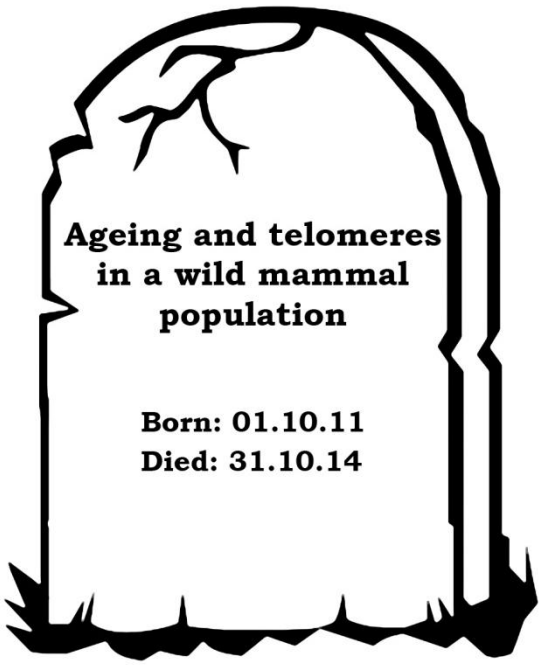
Table 1. Model selection on the factors affecting body condition (SMI) during the senescent period (≥ 5 years old). The final column indicates those models that were retained after the application of the nesting rule (Richards *et al.* 2011), whereby models are removed if they are more complex versions of nested (simpler) models that attracted stronger support. The grey area denotes the models included in the top set (after excluding models following the nesting rule), ✓ = categorical terms included in the model, * = interaction between two terms, Int = intercept, SGS = Social Group Size, ALC = Age at Last Capture, Age = Age in days, LYC = Last Year of Capture, df = degrees of freedom, AICc = Akaike's Information Criterion corrected for small sample size, ΔAICc = change in AICc relative to best supported model, AW = adjusted weight after removal of more complex models with less support.

Table 1. Model selection output for males experiencing low density. The output table for model selection on the factors affecting body mass during the senescent period (≥ 5 years old) where only males who experienced low early adulthood male density and all females are included. The final column indicates those models that were retained after the application of the nesting rule (Richards *et al.* 2011). The grey area denotes the models included in the top set (the second model in the table was excluded following the nesting rule), ✓ = terms included in the model, * = interaction between two terms, Int = intercept, SGS = Social Group Size, ALC = Age at Last Capture, Age = Age in days, Age_{cat} = Age coded categorically (years), LYC = Last Year of Capture, df = degrees of freedom, AICc = Akaike's Information Criterion corrected for small sample size, ΔAICc = change in AICc relative to best supported model, AW = adjusted weight after removal of more complex models with less support.

Int	Sex	Month	SGS	ALC	Age	Age^2	Age*Sex	Age^2*Sex	YLC	Sex*YLC	Age_{cat}	Age_{cat}*Sex	df	logLik	**Δ**AICc	AW	INC
8.96	-0.01	✓	-0.03	✓	0.33	-0.03			✓				22	-1329	0.00	0.71	✓
8.97	-0.01	✓	-0.03	✓	0.32	-0.02			✓	✓			23	-1329	1.77	0.00	
8.68	0.03	✓	-0.03	✓	0.30	-0.03							21	-1331	1.81	0.29	✓
9.02	-0.01	✓	-0.03	✓	0.32	-0.02	✓		✓				23	-1329	1.84	0.00	
8.73	0.03	✓	-0.03	✓	0.29	-0.03	✓						22	-1331	3.76	0.00	
9.01	-0.01	✓	-0.03	✓	0.31	-0.02	✓		✓	✓			24	-1329	3.77	0.00	
8.98	-0.01	✓	-0.03	✓	0.33	-0.03	✓	✓	✓				24	-1329	3.88	0.00	
8.67	0.03	✓	-0.03	✓	0.31	-0.03	✓	✓					23	-1331	5.72	0.00	
8.97	-0.01	✓	-0.03	✓	0.32	-0.03	✓	✓	✓	✓			25	-1329	5.81	0.00	
9.95	0.00	✓	-0.03	✓					✓		✓		28	-1326	6.38	0.00	
10.45	0.00	✓	-0.03	✓	-0.09				✓				21	-1334	7.10	0.00	
9.58	0.03	✓	-0.03	✓							✓		27	-1328	7.22	0.00	
9.94	0.00	✓	-0.03	✓					✓	✓	✓		29	-1326	8.20	0.00	
10.44	0.00	✓	-0.03	✓	-0.09				✓	✓			22	-1333	8.47	0.00	
10.19	0.04	✓	-0.02	✓	-0.12								20	-1336	9.04	0.00	
9.98	0.00	✓	-0.03	✓					✓		✓	✓	34	-1323	13.71	0.00	
9.62	0.03	✓	-0.03	✓							✓	✓	33	-1325	14.29	0.00	
9.97	0.00	✓	-0.03	✓					✓	✓	✓	✓	35	-1323	15.68	0.00	
10.53	-0.06	✓	-0.02	✓					✓				20	-1340	17.30	0.00	
10.51	-0.06	✓	-0.03	✓					✓	✓			21	-1339	18.26	0.00	
9.84	-0.02	✓	-0.02	✓									19	-1351	37.15	0.00	

Table 2. Model selection output for males experiencing high density. The output table for model selection on the factors affecting body mass during the senescent period (≥ 5 years old) where only males who experienced high early adulthood male density and all females are included. The final column indicates those models that were retained after the application of the nesting rule (Richards *et al.* 2011). The grey area denotes the models included in the top set (the second, third and fourth models in the table were excluded following the nesting rule), ✓ = terms included in the model, * = interaction between two terms, Int = intercept, SGS = Social Group Size, ALC = Age at Last Capture, Age = Age in days, Age_{cat} = Age coded categorically (years), LYC = Last Year of Capture, df = degrees of freedom, AICc = Akaike's Information Criterion corrected for small sample size, ΔAICc = change in AICc relative to best supported model, AW = adjusted weight after removal of more complex models with less support.

Int	Sex	Month	SGS	ALC	Age	Age^2	Age*Sex	Age^2*Sex	YLC	Sex*YLC	Age_{cat}	Age_{cat}*Sex	df	logLik	**Δ**AICc	AW	INC
8.99	-0.02	+	-0.02	+	0.35	-0.03	✓		✓				23	-1302	0.00	0.94	✓
8.99	-0.02	+	-0.02	+	0.35	-0.03	✓		✓	✓			24	-1302	1.91	0.00	
9.04	-0.02	+	-0.02	+	0.34	-0.02	✓	✓	✓				24	-1302	2.04	0.00	
9.04	-0.02	+	-0.02	+	0.34	-0.03	✓	✓	✓	✓			25	-1302	3.96	0.00	
8.64	0.02	+	-0.02	+	0.33	-0.03	✓						22	-1306	5.59	0.06	✓
8.69	0.02	+	-0.02	+	0.32	-0.03	✓	✓					23	-1306	7.60	0.00	
8.96	-0.02	+	-0.02	+	0.34	-0.03			✓	✓			23	-1309	12.78	0.00	
8.94	-0.02	+	-0.02	+	0.34	-0.03			✓				22	-1311	15.65	0.00	
10.12	-0.02	+	-0.02	+					✓		✓	✓	36	-1297	17.41	0.00	
10.13	-0.02	+	-0.02	+					✓	✓	✓	✓	37	-1296	18.11	0.00	
9.91	-0.01	+	-0.02	+					✓	✓	✓		29	-1306	19.94	0.00	
8.58	0.02	+	-0.02	+	0.32	-0.03							21	-1315	21.15	0.00	
10.61	-0.01	+	-0.02	+	-0.11				✓	✓			22	-1314	22.17	0.00	
9.62	0.02	+	-0.02	+							✓	✓	35	-1301	22.51	0.00	
9.91	-0.02	+	-0.02	+					✓		✓		28	-1309	23.46	0.00	
10.60	-0.02	+	-0.02	+	-0.11				✓				21	-1317	25.12	0.00	
9.43	0.02	+	-0.02	+							✓		27	-1312	27.94	0.00	
10.29	0.03	+	-0.02	+	-0.14								20	-1321	31.49	0.00	
10.61	-0.09	+	-0.02	+					✓	✓			21	-1324	39.31	0.00	
10.60	-0.10	+	-0.02	+					✓				20	-1326	41.13	0.00	
9.84	-0.05	+	-0.01	+									19	-1344	75.04	0.00	


Ageing and telomeres in a wild mammal population
Born: 01.10.11
Died: 31.10.14