

Early-life effects on telomere  
dynamics in European badgers  
(*Meles meles*)

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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**Ethical statement**

All work conducted in this thesis was approved by the University of Oxford's Animal Welfare and Ethical Review Board, ratified by the University of Leeds, and carried out under Natural England Licenses, currently 2017-27589-SCI-SCI and Home Office Licence (Animals, Scientific Procedures, Act, 1986) PPL: 30/3379.

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# Abstract

Despite extensive evidence of senescence, the decline in performance with age, in wild populations, the drivers of individual variation in senescence patterns are still unresolved. In this thesis, I study how early-life environmental, genetic and transgenerational effects contribute to individual variation in senescence patterns, using telomere dynamics, in a wild population of European badgers (*Meles meles*).

I discovered that telomere length forms a complex relationship with age, with both decreases and increases in telomere length that cannot be fully explained by measurement error. Telomere length was not sex-specific, but early-life telomere length predicts survival to adulthood (>1 year old) and lifespan. Within-individual changes in telomere length could be due to age-related changes in leukocyte cell composition in response to social conditions. While variation in (early-life) telomere length was associated with the abundance and variation in food availability, and natal but not adult social conditions, I found no evidence for heritability of telomere length or transgenerational effects, through parental age at conception effects. Additionally, individuals experiencing matching early- and later-life conditions had longer lifespans, even though there was only moderate autocorrelation in environmental quality, but this also depended on the mean environmental quality across adulthood. I also developed a novel approach to the analysis of long-term studies, termed slicing, which overcomes problems with confounding effects and cross-classified data structures.

My research shows that individual variation in telomere length and senescence is a consequence of early-life environmental, not genetic or transgenerational, effects in European badgers. In addition, I show the potential for adaptive responses in anticipation of the adult environment and the importance of studying both the mean of and variability in early-life conditions to fully understand the selective pressures on senescence.

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## Commonly used abbreviations

DNA: DeoxyriboNucleic Acid

MMqPCR: Monochrome Multiplex quantitative Polymerase Chain Reaction

RLTL: Relative Leukocyte Telomere Length

ROS: Reactive Oxygen Species

PAC: Paternal Age at Conception

MAC: Maternal Age at Conception

PAR: Predictive-Adaptive-Response

# Chapter 1: General introduction

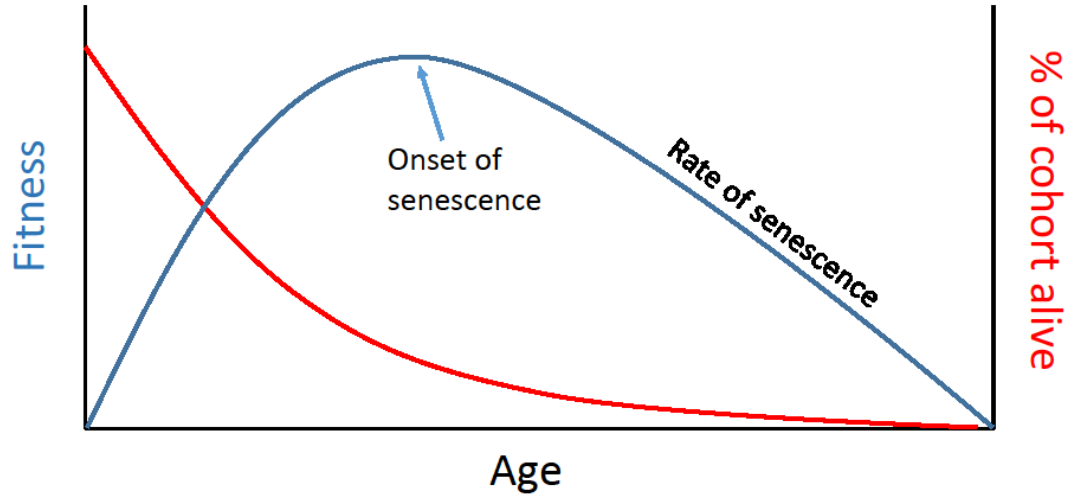
### **1.1 Organismal senescence**

Organismal senescence is the irreversible accumulation of damage with age that results in a loss of function and eventual death. This is often manifested with an increasing probability of mortality and decreasing reproductive success with age (Kirkwood and Rose, 1991; Stearns, 1992). Williams (1957) noted that while individuals are able to create a complex and functioning soma, they lack the ability to maintain what is already formed. Understanding how a process where progressive and irreversible changes impair rather than improve individual performance evolved has become an important aim in evolutionary biology.

### **1.2 Evolutionary theories of senescence**

The initial thought was that senescence benefits the population by removing old and unproductive individuals from the population, which accelerates the renewal of generations and facilitates adaptation to changing environments (Weissman, 1889; Wallace, 1865). However, senescence should be selected against as it does not benefit the individual, except in circumstances of kin or group selection (Bourke, 2007), and the initial thought was therefore superseded by the now classical evolutionary theories of senescence.

The foundation for the theories of senescence was laid down by Peter Medawar, who posited that even in the absence of senescence, the genetic mutations expressed in early-life have a higher impact on individual fitness than mutations expressed in later-life, due to the pressure of age-independent extrinsic mortality (Medawar, 1952). Hence, mutations expressed in early-life will be under stronger selection, since the proportion of individuals that are alive at progressively older ages declines (Figure 1.1). That forces of natural selection decline with age was later mathematically refined (Williams, 1957; Hamilton, 1966; Charlesworth, 1980).



**Figure 1.1: Senescence and the strength of natural selection. The age trajectory of an individual with fitness against age. After an increase in fitness in early-life, fitness starts to decline, defining the onset of senescence. The slope with which it declines is the rate of senescence. The forces of natural selection decline with age due to pressure of age-independent extrinsic mortality, with mutations in early-life under stronger selection since the number of individuals in a cohort declines with age.**

There are three main hypotheses explaining the evolution of senescence. The first hypothesis, termed ‘mutation accumulation’, states that under the mutation–selection balance, deleterious mutations accumulate over life and act in later-life. This results in a decrease in physiological functions, such as reduced survival and reproductive success, in older individuals (Medawar, 1952). Such mutations could still be favourable in early-life and therefore undergo positive selection. The existence of antagonistically pleiotropic genes, which have deleterious effects in later-life, are nonetheless favoured because of their positive effects on survival and reproductive success in early-life when selection is stronger. This is known as the ‘antagonistic pleiotropy hypothesis’ (Williams, 1957). The third hypothesis can be viewed as a physiological variant of the antagonistic pleiotropy hypothesis, termed the ‘disposable soma hypothesis’. This

hypothesis is based on trade-offs in allocation of limiting resources to self-maintenance and other activities, such as reproduction (Kirkwood, 1977; Kirkwood and Rose, 1991). The decline in organismal functioning results from the accumulation of somatic damage to cells as a result of physiological processes that produce harmful by-products and physiological stress (Sgro and Partridge, 1999; Westendorp and Kirkwood, 1998). The rate of accumulation of damage is influenced by biochemical mechanisms, such as antioxidants and DNA repair enzymes. These mechanisms require resources for self-maintenance which could have otherwise been allocated to other activities such as reproduction or avoiding factors contributing to extrinsic mortality. The deterioration of an individual therefore reflects the balance in resource allocation to self-maintenance and other activities (Kirkwood, 2005). All three mutually non-exclusive hypotheses provide potential explanations for the evolution of senescence, but in contrast to 'mutation accumulation', the 'antagonistic pleiotropy' and 'disposable soma' hypotheses are adaptive since selection depends on beneficial traits and trade-offs between early- and later-life. However, it was long considered that these hypotheses were only applicable to human populations and not to wild organisms as, due to extrinsic mortality, they would not survive to ages at which senescence is observed (Medawar, 1952; Kirkwood and Austad, 2000).

### **1.3 Senescence in the wild**

Empirical evidence for senescence in wild animals has accumulated, with 340 studies of over 175 species that provide evidence of senescence in the wild (Nussey et al., 2013). This opposes the fallacy that animals in wild populations do not senesce (Medawar, 1952), and supports the view that senescence is common in wild species. Understanding senescence in the wild is important because, in contrast with studies in laboratory conditions, selection acts on multiple traits simultaneously. These phenotypic traits evolve under natural selection, where variable

environments may influence genes that lead to age-dependent phenotypic variation and in turn shape fitness in a natural setting (Charmantier et al., 2014; Partridge and Gems, 2007). However, among these wild populations there are species that are not currently thought to show any evidence of senescence. For example, naked mole-rats (*Heterocephalus glaber*) defy the Gompertzian law by showing no increase in the probability of mortality at older ages (Ruby et al., 2018). It is currently not understood why some species have evolved patterns of senescence while other species may not have. For species that do exhibit senescence, there is considerable variation between species (Jones et al., 2014) and within species (e.g. Dugdale et al., 2011b; Nussey et al., 2009) in the onset and rate of senescence, and current studies focus on determining factors that explain this individual variation in senescence patterns.

#### **1.4 Factors contributing to individual variation in senescence patterns**

There is increasing understanding about how natural variation in extrinsic environments shapes individual senescence patterns (Cooper and Kruuk, 2018; Nussey et al., 2013; Lemaitre and Gaillard, 2017). Since an optimal distribution of resources within individuals is favoured by selection, this subsequently optimises individual fitness (Stearns, 1992). Thus, whenever reproduction is favoured at the expense of somatic investment, individuals will senesce more rapidly (Kirkwood and Rose, 1991). For example, female red deer (*Cervus elaphus*) that produced more offspring in early-life showed faster rates of reproductive senescence (Nussey et al., 2006), whereas males with larger harems and therefore more resource investment in reproduction in early adulthood exhibited faster rates of senescence in harem size and rut duration (Lemaitre et al., 2014). Additional empirical evidence for trade-offs between early-life reproduction and somatic maintenance exists in wild birds (Charmantier et al., 2006; Reed et al., 2008).

Trade-offs between reproductive and somatic investment also differ between the sexes. For example, male European badgers (*Meles meles*) show a later onset but faster rate of senescence than females (Dugdale et al., 2011b). An explanation for such patterns initially stems from anisogamy (Bateman, 1948; Trivers, 1972), and that in polygynandrous species males benefit from numerous matings, whereas females have fewer reproductive benefits from many matings and show less risky behaviours (Bonduriansky et al., 2008; Singh and Punzalan, 2018). Males may therefore exhibit greater intra-sexual competition than females, which results in a male-biased mortality rate (Bonduriansky et al., 2008; Maklakov and Lummaa, 2013; Promislow, 2003). This could result in a differential weakening of the forces of selection against deleterious mutations or antagonistic pleiotropic genes between the sexes, leading to faster rates of senescence in males than in females (Andersson, 1994; Williams, 1957; Hamilton, 1966). A study in a wild population of European badgers, which exhibit male-biased mortality (McDonald et al., 2014), supports this view because males exhibited higher rates of body mass senescence due to greater intra-sexual competition in early adulthood (Beirne et al., 2015).

Senescence rates have also been hypothesised to be faster in adverse early-life environments which induce high mortality rates (Williams, 1957), such as greater resource competition or lower food availability (Nussey et al., 2007a; Hammers et al., 2013; Campbell et al., 2017; Douhard et al., 2016). Besides these effects of the mean environmental conditions, the variation around this mean among years and variability within years could shape senescence patterns (Nussey et al., 2007a; Reid et al., 2003). Individuals living in temporally stochastic environments should adopt a bet-hedging strategy with higher somatic than reproductive investment (Morris et al., 2008; Wilbur and Rudolf, 2006; ; but see Shpak, 2007), which results in longer lifespans in variable conditions. However, variable environmental conditions can lead to maladaptation and may have age-specific effects, due to divergent strengths of natural

selection, that differentially affect genotypes, causing faster senescence rates in variable environmental conditions (Cotto and Ronce, 2014).

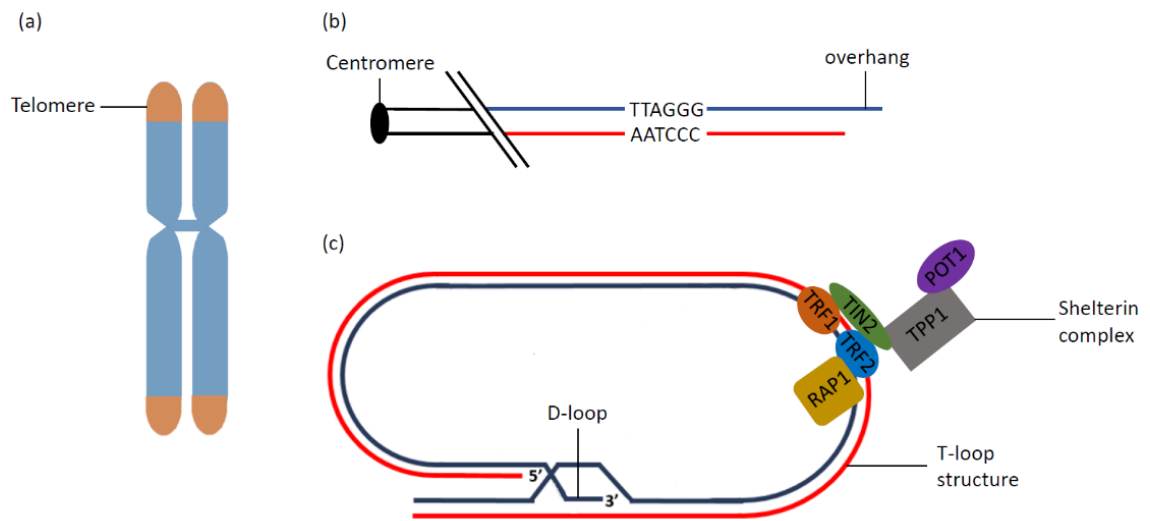
Variation in individual responses to environmental conditions provides another source for variation in senescence patterns. These plastic responses in morphological and life-history traits can be the result of a differential investment in reproduction or the soma, and are fundamental for a population to cope with environmental change (Nussey et al., 2007b). However, individuals can be physiologically constrained to provide a plastic response (Ricklefs and Wikelski, 2002; Bateson et al., 2004). For individuals experiencing similar environments, this might result in an individual-specific phenotypic response that shapes individual life-history and therefore senescence patterns (Ricklefs and Wikelski, 2002). Additionally, constraints in adaptation may also depend on the similarities in experienced environments throughout life (Bateson et al., 2004; Gluckman et al., 2005a; Nettle et al., 2013a). For example, individuals born in favourable conditions may always outperform those born in adverse conditions, known as the 'silver spoon hypothesis' (Lindstrom, 1999; Douhard et al., 2014; van de Pol et al., 2006; Cooper and Kruuk, 2018; Grafen, 1988). However, a mismatch between early- and later-life environmental conditions, and therefore incorrectly predicting the adult environment, adaptive strategy and response, can result in detrimental effects on individual life-history, known as predictive adaptive response (PAR) hypotheses (Nettle et al., 2013a).

Even though there is increasing understanding of the factors that contribute to individual variation in senescence patterns, the effects of environmental conditions can be difficult to measure. Understanding the consequences of such conditions on individual senescence patterns can be improved by quantification of these extrinsic conditions through biomarkers.



## 1.5 Telomeres

A biomarker which reflects the physiological consequences of within-individual experiences and facilitates between-individual comparisons is telomere length (Monaghan and Hausmann, 2006). Telomeres are non-coding hexameric repeats (5'-TTAGGG-3') which form a nucleoprotein structure with associated shelterin complexes (Blackburn, 1991; Figure 1.2). These structures are located at interstitial chromosome sites and at the terminal ends of all eukaryotic chromosomes, where they prevent end-to-end fusion of linear chromosomes through the formation of t-loops and maintain genomic integrity (Blackburn, 2000; de Lange, 2004). Telomeres shorten with age due to successive cell divisions where DNA-replication at the 3'-end of the DNA-strand is incomplete, known as the end-replication problem (Olovnikov, 1973). Consequently, telomeres shorten more rapidly in early-life due to higher levels of cell division during growth (Frenck et al., 1998; Hall et al., 2004). However, other metabolically demanding activities such as reproduction, disease and stress also contribute to the rate of change in telomere length (Heidinger et al., 2012; Epel et al., 2004; Willeit et al., 2010). The amount of telomeric DNA lost in each cell division also depends on the cellular conditions (Monaghan and Ozanne, 2018). While *in vitro* evidence exists that oxidative stress contributes to telomere shortening (von Zglinicki, 2002; Richter and von Zglinicki, 2007), whether oxidative stress is a mechanism that explains telomere shortening *in vivo* remains currently unclear (Reichert and Stier, 2017; Boonekamp, 2017).



**Figure 1.2: Structure of telomeres: a) a chromosome before cell division with telomeres at the terminal ends; b) the linear telomere structure and sequence in eukaryotic chromosomes, where the DNA-strand has an overhang at the 3'-end due to the end-replication problem; and c) the telomere T-loop that prevents fusion of linear chromosomes with the single strand invading the double stranded telomeric DNA (D-loop). The shelterin complex aids in T-loop formation and telomere functioning and thus maintaining genomic integrity.**

## 1.6 Oxidative stress

Oxidative stress, which originated from the free radical theory of ageing, is potentially a contributing factor to the shortening of telomeres (Wickens, 2001). During oxygen metabolism in the mitochondria, as a by-product, reactive oxygen species (ROS) are produced which can cause cellular damage (Selman et al., 2012; Beckman and Ames, 1998). Within telomeres the most prevalent nucleobase guanine is especially sensitive to oxidation by ROS (Oikawa and Kawanishi, 1999). Although ROS can be neutralized by antioxidants, reactive molecules that are not will induce oxidative stress (Finkel and Holbrook, 2000; Costantini and Verhulst, 2009). While there is evidence from laboratory studies that oxidative stress contributes to telomere shortening (Liu et al., 2002), where the level of oxidative stress can be modified by both genetic

and environmental conditions (Harman, 1992; Martin et al., 1996), it remains currently unclear if this occurs in whole organisms (Reichert and Stier, 2017; Boonekamp, 2017).

Oxidative stress provides a potential pathway by which adverse conditions can detrimentally affect telomere length. A proximate mechanism that could mediate this link between the adverse environment and telomeres, through the stress response, are glucocorticoids (Angelier et al., 2018). The response to unpredictable events that induce stress is the secretion of glucocorticoids (Romero, 2004). Elevated levels of glucocorticoids are positively linked with oxidative stress (Costantini et al., 2011), which could result in greater DNA damage and shorter telomere lengths when energy investment promotes immediate survival at the expense of demanding other activities. However, such elevation of telomere shortening only occurs when telomerase is inactive (Choi et al., 2008).

### **1.7 Telomerase, alternative lengthening pathways and cellular senescence**

Telomeres can be restored or even elongated by telomerase, the telomere-elongating enzyme, which is highly prolific in stem cells (Blackburn et al., 1989). Even though telomerase activation can slow-down genomic instability from dysfunctionally short telomeres (Kim et al., 1994), it can also confer immortality on cells which increases the likelihood of cancer (Robin et al., 2014; Kim et al., 2016). This is why telomerase is transcriptionally repressed later in development (Blackburn et al., 1989) and other tumour suppression mechanisms (Peto, 2015; Caulin and Maley, 2011; Risques and Promislow, 2018), for example additional p53 pseudogenes (Vazquez et al., 2018), have evolved. However, alternative pathways for telomere lengthening do exist (Cesare and Reddel, 2010; Mendez-Bermudez et al., 2012).

Another potential explanation for telomere lengthening in mammals, in contrast with birds, is that telomere length is measured from DNA in leukocytes. Since granulocytes have

longer telomeres than agranulocytes in humans and baboons (Baerlocher et al., 2007; Kimura et al., 2010), a change in leukocyte cell composition with age may appear as a positive change in mean telomere length and thus be inferred as telomere lengthening. Leukocytes are involved in the immune system, and environmental conditions may shape sex-specific leukocyte cell compositions (Klein and Flanagan, 2016; Restif and Amos, 2010). The immune system can be broadly divided into the innate and adaptive immune responses, where declines in performance of the adaptive immune response (i.e. immunosenescence) occur in wild animals (Nussey et al., 2012; Palacios et al., 2011; Schneeberger et al., 2014; Peters et al., 2019; Cheynel et al., 2017). Such immunosenescent declines are the consequence of microbial exposure, nutrition and the developed microbiome (McDade, 2012; Klein and Flanagan, 2016), and may be subject to other environmental conditions which can lead to the appearance of telomere lengthening but is merely a change in leukocyte cell composition. Telomere shortening occurs until cells enter a state of arrest, inducing replicative senescence, where the accumulation of senescent cells, due to progressive loss of regenerative capacity (Campisi and di Fagagna, 2007), can impair tissue functioning (Campisi, 2005; Armanios and Blackburn, 2012).

### **1.8 Telomere length and life-history**

Telomere length is a biomarker of senescence (Monaghan and Haussmann, 2006; Wilbourn et al., 2018), that may reflect an individual's somatic state linked to the cumulative stress that the individual has experienced during its life (Monaghan, 2010). Telomere length has been associated with life-history parameters, such as survival to adulthood, annual adult survival probability and lifespan in both captive and wild populations (Wilbourn et al., 2018). Even though some studies tested for, but did not find such relationships (Beaulieu et al., 2011; Sudyka et al., 2014), a meta-analysis in non-human vertebrates reported an overall association between short

telomeres and a higher mortality risk (Wilbourn et al., 2018). However, it remains currently unclear whether telomere length plays a direct causal role in senescence, where short telomeres induce senescence, or whether telomeres reflect the damage to other biological structures that have deleterious effects on fitness (Simons, 2015; Young, 2018). Nevertheless, the challenge is to determine the genetic, epigenetic and environmental factors that generate individual variation in telomere length and their implications for life-history strategies and senescence.

### **1.9 Intrinsic and extrinsic factors linked to telomere length**

Since sex differences in life-history and senescence patterns occur (Bonduriansky et al., 2008; Beirne et al., 2015) these sex differences are expected to be found in telomere length. In humans and laboratory rodents, adult females have longer telomere lengths than adult males (Barrett and Richardson, 2011; Gardner et al., 2014), which correlates with the sex-specific differences in lifespan in these species (Stindl, 2004). Explanations for such sex differences in telomere length could be that in species with sexual dimorphism there is increased cell proliferation in the larger sex, heterogametic disadvantage or effects of sex hormones (Stindl, 2004; Barrett and Richardson, 2011). The development of secondary sexual characteristics requires an additional energetic investment which, for example, in polygamous males may result in faster telomere shortening (Andersson, 1994; Monaghan, 2010). These results indicate that in species with similar lifespans in males and females no sex difference in telomere length exists (e.g. Cram et al., 2017), whereas in species with sex differences in lifespan and development of secondary characteristics age-dependent sex differences in telomere length can be found (Watson et al., 2017).

Adverse early-life environmental conditions can have deleterious effects on telomere length. Adverse early-life conditions usually require additional energy investment, and in

combination with faster growth in early-life this can exponentially shorten telomeres. There is extensive evidence that increased conspecific competition for resources among juvenile siblings is reflected in shorter telomere length (Cram et al., 2017; Nettle et al., 2015; Boonekamp et al., 2014; Stier et al., 2015). This finding is supported by other studies using different metrics for environmental quality (Mizutani et al., 2013; McLennan et al., 2016; Watson et al., 2015; Wilbourn et al., 2017), where a lower environmental quality in early-life was reflected in shorter telomeres.

Social cues in early-life may also change the trade-off between reproductive investment and somatic maintenance, through developmental plasticity that may be reflected in early-life telomere length (Bretman et al., 2016; Kasumovic and Brooks, 2011). The differential effects of early-life conditions also become apparent through the strong cohort effects found in studies that link telomere length with age, indicating that early-life conditions are reflected in individual telomere length and dynamics (Fairlie et al., 2016; Spurgin et al., 2017).

Besides environmental conditions, transgenerational effects, such as parental age at conception, can also affect telomere length (Eisenberg and Kuzawa, 2018). Paternal, but not maternal, age at conception is predicted to affect offspring telomere length because sperm is produced continuously whereas oocytes are produced prenatally (Eisenberg and Kuzawa, 2018). A positive relationship between paternal age at conception and offspring telomere length has been reported in humans (Hjelmborg et al., 2015; Kimura et al., 2008; Njajou et al., 2007) and chimpanzees (Eisenberg et al., 2017). Explanations for this positive effect is that telomerase activity in the germ line is high to compensate for telomere loss, and might overshoot the original telomere length (Kimura et al., 2008; Aviv and Susser, 2013). Another explanation is that there is selective loss of germ cells with shorter telomeres, making the stem cells with longer telomeres more abundant (Kimura et al., 2008; Hjelmborg et al., 2015). However, studies into the

associations between paternal or maternal age at conception effects and offspring telomere length in other taxa have shown parental age effects in different directions (Noguera et al., 2018; Dupont et al., 2018; Bouwhuis et al., 2018; Eisenberg and Kuzawa, 2018; Asghar et al., 2015a; Olsson et al., 2011; Froy et al., 2017). These different results might be related to the mating system in the associated species (Bouwhuis et al., 2018).

The proportion of additive genetic variance out of the total phenotypic variance in telomere length defines whether telomere length is heritable. With the associated strength of selection, this theoretically indicates the evolutionary potential of a trait such as telomere length (Lynch and Walsh, 1998). Heritability of telomere length has been determined in a variety of species (Olsson et al., 2011; Horn et al., 2011; Voillemot et al., 2012; Reichert et al., 2015), however this is usually based on parent–offspring regressions which cannot decompose shared environments between offspring and parents (Dugdale and Richardson, 2018). Three recent studies in non-human vertebrates have used the ‘animal model’, an extension of the mixed model that takes into account relatedness, providing no or high heritability estimates of telomere length in semi-feral cattle and wild birds (Seeker et al., 2018; Becker et al., 2015; Asghar et al., 2015a).

Most studies into telomere dynamics in wild populations come from avian studies. However, studies into the effects of environmental conditions on individual telomere length are emerging in wild mammals (Izzo et al., 2011; Lewin et al., 2015; Cram et al., 2017; Beirne et al., 2015), but longitudinal studies remain relatively rare (Fairlie et al., 2016; Beirne et al., 2014). Longitudinal studies are required for interpretation of within-individual telomere length as, for example, recent evidence suggests that telomere length can both increase and decrease with age, independent of any measurement error (Spurgin et al., 2017). Studying the association between telomere length and life-history and any contributing genetic, environmental and

parental effects in divergent taxa will improve understanding of individual variation in telomere length and senescence patterns.

### **1.10 Structuring samples for analysis in long-term studies**

Understanding the relationships between telomere dynamics, life-history strategies and environmental conditions requires long-term studies. These studies measure individuals repeatedly across their lifetimes and among generations, providing data over adequate periods of time (Clutton-Brock and Sheldon, 2010). However, the statistical analyses of such comprehensive biological datasets can be complex due to the hierarchical nature of biology. When all observations fall within a single observation at a higher hierarchical level, a nested design, this can be analysed with statistical mixed models (Gelman and Hill, 2006; Bolker et al., 2009). However, a discrepancy between the longitudinal collection of data and the associated laboratory analysis of, for example, blood samples for telomere length, often results in cross-classified data structures. These structures arise when observations are associated with multiple observations at a higher hierarchical level, and require a different interpretation of the statistics and variance components (Schielzeth and Nakagawa, 2013). Cross-classified data structures can be turned into a nested design when longitudinal samples from a single individual are analysed in the same batch (e.g. qPCR-plate), but this results in complicated re-ordering of samples and the potential for human error (Beirne et al., 2014; Nettle et al., 2015). Additionally, the intervals between laboratory analyses in the same long-term study can result in confounding effects with current approaches such as sequential analysis or randomisation (Takizawa et al., 2004; Spurgin et al., 2017; Swanson et al., 2015; Dantzer et al., 2013). The often used randomisation approach in long-term studies confounds analyses that are randomised separately, which commonly occurs, and does not allow flexible inclusion of currently analysed data in future analyses. The



approach to structuring samples for analysis in long-term studies and the associated statistical analyses requires consideration to cope with cross-classified data structures and confounding effects.

### **1.11 Study species: European badgers (*Meles meles*)**

This thesis examines the relationships between environmental, genetic and parental effects and telomere length and associated life-history in a wild population of European badgers (*Meles meles*). European badgers are medium-sized carnivores that belong to the mustelid family (Kruuk, 1978a). Badgers are primarily nocturnal and live in underground dens, termed setts (Thornton, 1988). They are a facultatively social species and form large social groups (mean group size: 11.3, range 2–29; da Silva et al., 1994; Macdonald et al., 2015) in high-density populations (Johnson et al., 2000). Badger social groups, a collection of setts, have clearly demarcated territories (Buesching et al., 2016; Delahay et al., 2000), although badgers do cross these borders when foraging and meet amicably with neighbouring groups (Ellwood et al., 2017; Noonan et al., 2015).

In high-density populations, European badgers have a polygynandrous mating system (Dugdale et al., 2011a). Multiple breeding males and females are present in each social group, but reproductive skew exists among males and females within a social group (Dugdale et al., 2008). Reproductive activity occurs during a post-partum oestrus, and while matings can occur throughout the year a secondary mating peak exists in late summer (Buesching et al., 2009; Sugianto et al., 2019b). Mating and pregnancy are decoupled because females exhibit embryonic diapause, where the fertilised eggs cease development and are implanted in mid-winter (Yamaguchi et al., 2006). Female badgers are also capable of conception during pregnancy (i.e. superfetation), and within a single litter cubs can be sired by different fathers due to fertilisation

of a second ovum (i.e. superfecundation; Yamaguchi et al., 2006). Implantation occurs in mid-winter with changes in photo-period (Canivenc and Bonnin, 1981) and cubs are born in February, with a mean litter size of 1.4 ( $\pm 0.06$  SE; range = 1–4; Dugdale et al., 2007). Cubs emerge from the underground den at 6–8 weeks old, are weaned at 12 weeks and reach independence at 14–16 weeks (Fell et al., 2006). Badgers exhibit two developmental strategies, with early developers reaching sexual maturity at 11 months old and late developers at 22–26 months old (Sugianto et al., 2019b). While cub growth rates and maturation depend on natal social conditions, these effects appear to be stronger for male cubs (Sugianto et al., 2019b; Sugianto et al., 2019a). Dispersal from the natal social group is low and within-group relatedness is high therefore, as a mechanism to reduce inbreeding, high rates of extra-group paternity occur (48%; Annavi et al., 2014b; Dugdale et al., 2007) which increases with fewer within-group candidate fathers.

Male badgers experience density-dependent increases in bite wound frequency (Macdonald et al., 2004a). There is male-biased sexual dimorphism (Johnson and Macdonald, 2001) and male-biased mortality (Bright Ross, unpublished data). Badgers mainly feed on earthworms (*Lumbricus terrestricus*; Johnson et al., 2001; Kruuk and Parish, 1981); thus, high badger densities occur in areas with mild and damp conditions where earthworms are available (Kruuk, 1978a; Macdonald et al., 2015; Newman et al., 2017). Earthworms are highly sensitive to microclimatic conditions (Edwards and Bohlen, 1996; Gerard, 1967; Newman et al., 2017), making their abundance highly dependent on weather patterns. While badgers preferentially eat earthworms, they do change to other food sources when earthworms are scarcely available (Macdonald et al., 2010).

Badgers undergo reproductive senescence, where the onset of reproductive senescence is later and steeper in males compared to females (Dugdale et al., 2011b). Additionally, male badgers show higher body mass senescence than females which is attributable to downstream

effects of intra-sexual competition in early adulthood (Beirne et al., 2015). While badgers showed within-individual age-related declines in telomere length, these sex differences in senescence were not reflected in telomere length or the rate of shortening (Beirne et al., 2014). Badgers are detrimentally affected by early-life exposure to coccidia infection (Newman et al., 2001), oxidative stress (Bilham et al., 2018) and unseasonable weather variation (Macdonald et al., 2010; Noonan et al., 2014; Nouvellet et al., 2013). These detrimental effects may be linked to telomere length and therefore shape senescence patterns.

### **1.12 Study site and trapping**

Wytham Woods, Oxfordshire, UK (51°46'24"N, 1°20'04"W) is a 424 ha mixed semi-natural woodland site surrounded by mixed arable and permanent pasture (Macdonald and Newman, 2002; Macdonald et al., 2004b; Savill, 2010). The resident badger population has been continuously trapped since 1987, providing a life-history dataset on approximately 1600 badgers. The badger population density has increased since 1987 to a relatively constant current population density (range = 20.5–49.5 badgers/km<sup>2</sup>; Macdonald et al., 2015). This site has a mean of 19±2 (95% CI; range = 14–26; Dugdale et al., 2008) mixed-sex social groups (Johnson et al., 2002; Newman et al., 2011). The residing badger population is discrete geographically with a stable range (Macdonald et al., 2009) and <3% annual immigration/emigration (Macdonald and Newman, 2002). Cub survival probability ranges from 0.61–0.94 (mean±SE = 0.67±0.03; Macdonald et al., 2009), with a mean annual adult survival rate in this population of 0.83 (±0.01 SE; Macdonald et al., 2009) and a mean lifespan of 3.31 years (±3.51 SD; Bright Ross, J., Pers. Comm.). The annual recapture rate is 84% (SE = 1.3%; Macdonald et al., 2009).

Trapping was undertaken three or four times per year since 1987, over two weeks in May–June (Spring), August–September (Summer) and November (Autumn) with trapping in

January (Winter) in focal years. Badgers were trapped for two to three consecutive days per social group, with trapped badgers anaesthetised using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren et al., 2005) and identified by a unique tattoo number on the left inguinal region. Capture date, sett, social group (comprising several setts, i.e. burrow systems), sex, age-class (cub <1 year old; adult  $\geq$ 1 year old) and morphometric measurements (i.e. length, weight, tooth wear; da Silva and Macdonald, 1989; Macdonald et al., 2009) were recorded for each badger. The age of a badger was defined as the number of days elapsed since the 14<sup>th</sup> of February in their respective birth year (reflecting the February birth peak; Yamaguchi et al., 2006). Badgers first caught as adults were aged through tooth wear, with a tooth wear 2 indicating a 1-year old adult (da Silva and Macdonald, 1989; Macdonald et al., 2009). Blood was collected by jugular venipuncture into vacutainers with an EDTA anticoagulant and stored at -20°C immediately. Badgers were released at their setts, after full recovery from anaesthesia. Bait-marking was conducted periodically to delimit group size ranges and calculate group sizes (Delahay et al., 2000; Annavi et al., 2014b; Macdonald et al., 2008).

### **1.13 Thesis aim and outline**

In this thesis, I aim to examine the factors that contribute to individual variation in senescence patterns through telomere length as a biomarker of senescence, with extensive data on a wild population of European badgers (*Meles meles*; Figure 1.3). Specifically, I aim to: *i*) explore the link between telomere length and age, sex and life-history, and examine within-individual telomere dynamics; *ii*) investigate the change in immune cell profiles with age and any sex-specific associations with group size; *iii*) examine whether and how early-life intra-sexual group sizes and resource availability are reflected in early-life telomere length; *iv*) investigate parental age at conception effects and the contribution of additive genetic variance to the phenotypic

variance in telomere length; v) examine the importance of early-life conditions on fitness and the developing soma and the potential for adaptive life-history strategies in anticipation of the adult environment; and, vi) explore cross-classified data structures and confounding effects which are introduced by structuring samples for molecular analyses in long-term studies, and develop a novel approach for such analyses. I describe each of these chapters briefly below:

**Chapter 2** provides a comprehensive picture of the relationship between telomere length and age, telomere length and sex, within-individual telomere dynamics, whether any within-individual increase in telomere length is due to measurement error, and how telomere length is related to individual life-history in European badgers.

**Chapter 3** explores the change in leukocyte cell composition with age as a possible explanation for increases in telomere length with age, and examines whether leukocyte cell composition is associated with sex-specific responses to social group size.

**Chapter 4** investigates individual variation in early-life telomere length and whether effects of intra-sexual group sizes in early adulthood, natal group sizes and early-life weather conditions, as a proxy for resource availability, are reflected in early-life telomere length.

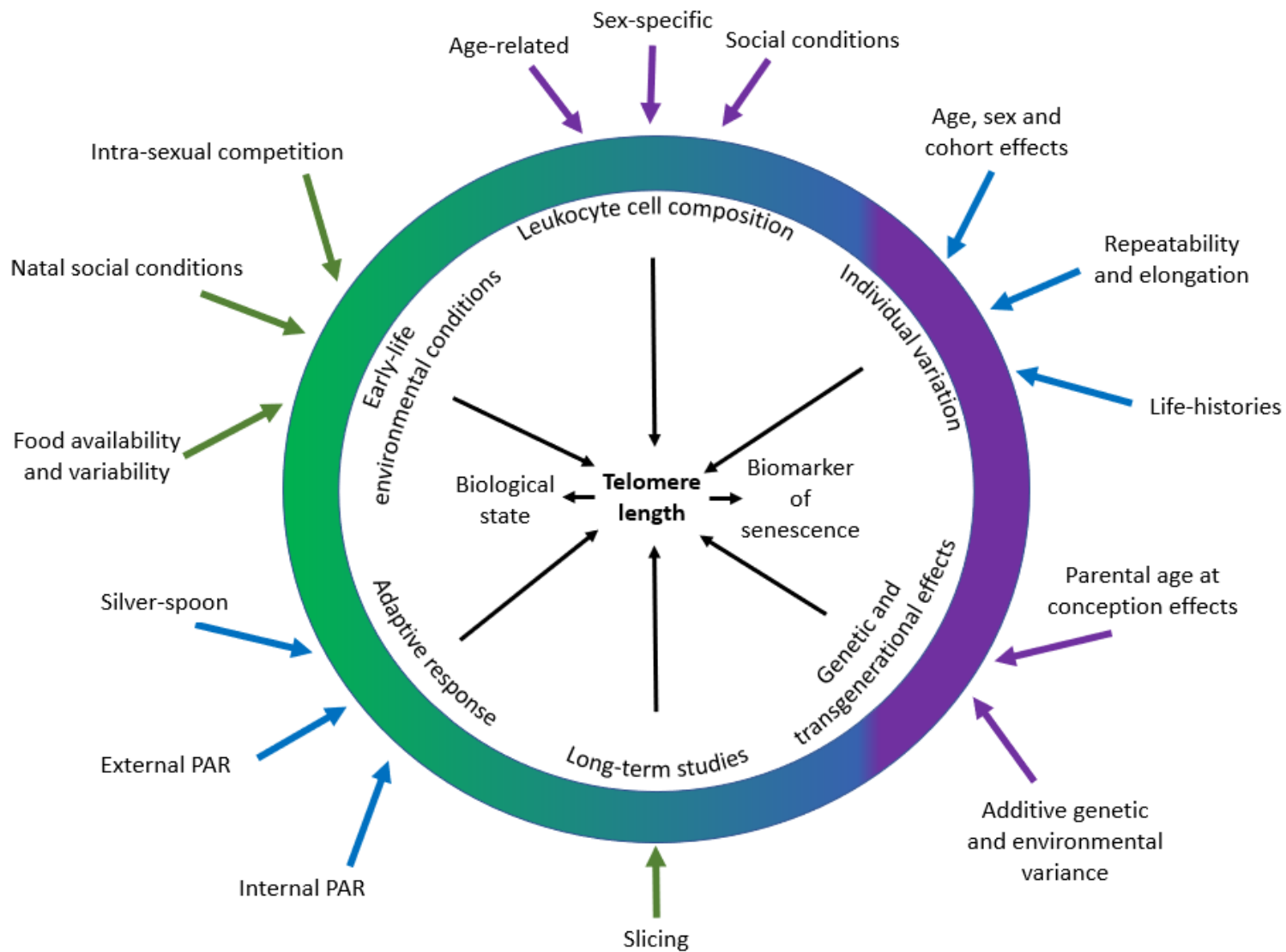
**Chapter 5** examines parental age at conception effects on offspring telomere length. Additionally, it explores how phenotypic variance in telomere length is separated into additive genetic and environmental variance using the 'animal model'.

**Chapter 6** explores whether silver-spoon effects are reflected in lifespan, and whether individuals are capable of an adaptive response based on the anticipated future somatic state (i.e. internal PAR). Additionally, it tests whether adaptive life-history strategies occur based on the similarity between the natal environment and adult environment (i.e. external PAR).

**Chapter 7** examines the potential for confounding effects and cross-classified data structures in long-term studies which arise through the inability to separate variation in variables

of interest from confounding effects, and describes a novel method to structuring samples for analyses that overcomes these issues.

Finally, **Chapter 8** provides a general discussion and consideration of the work contained within this thesis.



**Figure 1.3: Schematic of PhD chapters. Representation of specific factors within fields that may be linked to telomere length and therefore an individual's biological state or senescence patterns.**

## **Chapter 2: Individual variation in early-life telomere length and survival in a wild mammal**

*The following paper, van Lieshout S.H.J., Bretman, A., Newman, C., Buesching, C.D., Macdonald, D.W.M, Dugdale, H.L. (2019) Individual variation in early-life telomere length and survival in a wild mammal. Molecular Ecology, 28, 4152 – 4165, is a modified version of this chapter.*

I was responsible for conception and development of the study and I conducted laboratory work, statistical analyses and manuscript writing. Co-authors provided advice on data analysis and comments on draft manuscripts.



## 2.1 Abstract

Individual variation in survival probability due to differential responses to early-life environmental conditions is important in the evolution of life-histories and senescence. A biomarker allowing quantification of such individual variation, and which links early-life environmental conditions with survival by providing a measure of conditions experienced, is telomere length. Here, we examined telomere dynamics among 24 cohorts of European badgers (*Meles meles*). We found a complex cross-sectional relationship between telomere length and age, with no apparent loss over the first 29 months, but with both decreases and increases in telomere length at older ages. Overall, we found low within-individual consistency in telomere length across individual lifetimes. Importantly, we also observed increases in telomere length within individuals, which could not be explained by measurement error alone. We found no significant sex differences in telomere length, and provide evidence that early-life telomere length predicts lifespan. However, while early-life telomere length predicted survival to adulthood ( $\geq 1$  year old), early-life telomere length did not predict adult survival probability. Furthermore, adult telomere length did not predict survival to the subsequent year. These results show that the relationship between early-life telomere length and lifespan was driven by conditions in early-life, where early-life telomere length varied strongly among cohorts. Our data provide evidence for associations between early-life telomere length and individual life-history, and highlight the dynamics of telomere length across individual lifetimes due to individuals experiencing different early-life environments.

## 2.2 Introduction

Species from most taxa exhibit a loss of performance in later-life that increases the probability of mortality (Medawar, 1952; Williams, 1957). This process of senescence is common, but highly variable across taxa (Jones et al., 2014) and even within species (Campbell et al., 2017; Dugdale

et al., 2011b; Nussey et al., 2009). Pioneering laboratory studies using controlled environments have provided important insights into senescence patterns, but cannot explain the remarkable variation in the onset and rate of senescence in wild populations, where selection acts under naturally varying conditions (Partridge and Gems, 2007). Hence, studies of wild populations have informed understanding of how early-life environments shape individual senescence patterns (Cooper and Kruuk, 2018; Lemaitre et al., 2015; Nussey et al., 2013). This understanding has been further improved by quantification of extrinsic effects through biomarkers that reflect ecological effects that are otherwise difficult to measure (Bebbington et al., 2016; Spurgin et al., 2017).

Telomere length, which reflects the physiological consequences of within-individual experiences and facilitates between-individual comparisons, is a biomarker of senescence (Monaghan and Haussmann, 2006). Telomeres are non-coding hexameric repeats (5'-TTAGGG-3') that, with associated shelterin proteins, prevent end-to-end fusion of linear chromosomes and maintain genomic integrity (Blackburn, 2000; de Lange, 2004). Telomeres shorten with age due to incomplete DNA-replication at the 3'-end of the DNA-strand (Olovnikov, 1973). This occurs more rapidly in early-life due to higher levels of cellular division during growth (Frenck et al., 1998; Hall et al., 2004), or in response to metabolically demanding activities (e.g. reproduction; Heidinger et al., 2012; coping with stress/disease; Epel et al., 2004; Willeit et al., 2010). The amount of telomeric DNA lost in each cell division depends on cellular conditions (Monaghan and Ozanne, 2018) and oxidative stress (Reichert and Stier, 2017; von Zglinicki, 2002; but see Boonekamp, 2017). Telomeres can, however, be replenished by telomerase, the telomere-elongating enzyme (Blackburn et al., 1989). Telomerase is transcriptionally repressed later in development (Blackburn et al., 1989), but alternative pathways for telomere lengthening do exist (Cesare and Reddel, 2010; Mendez-Bermudez et al., 2012). Telomere shortening occurs until cells enter a state of arrest, inducing replicative senescence, where the accumulation of

senescent cells, due to progressive loss of regenerative capacity (Campisi and di Fagagna, 2007), can impair tissue functioning (Armanios and Blackburn, 2012; Campisi, 2005).

Variation in the rate of telomere shortening occurs among organisms (Monaghan, 2010). For example, mean human leukocyte telomere length shows a biphasic decline with age, with rapid shortening in early-life followed by slower attrition in adulthood (Aubert and Lansdorp, 2008). Correlations among within-individual telomere measurements in humans were high (0.82–0.93; Benetos et al., 2013), which corroborates the high individual repeatability (i.e. 81–83%) in telomere length in wild populations using TRF (telomere restriction fragment) methods (Bauch et al., 2013; Boonekamp et al., 2014). However, longitudinal studies in wild populations using a qPCR (quantitative-PCR) approach across individual lifetimes reported much lower (i.e. 7–13%) individual repeatability in telomere length (Fairlie et al., 2016; Spurgin et al., 2017), indicating that telomeres are highly dynamic over individual lifetimes. Indeed, telomere length can both decrease and increase with age (Bateson and Nettle, 2016), which has been attributed to measurement error (Steenstrup et al., 2013) but cannot be explained by measurement error alone (Spurgin et al., 2017). Telomere length can therefore exhibit complex relationships with age, explained by within-individual changes, and provide a measure of conditions experienced that links to individual life-history.

Telomere length has been linked positively to survival to adulthood and/or annual adult survival probability in both captive (Heidinger et al., 2012) and wild populations (Asghar et al., 2015b; Barrett et al., 2013; Cram et al., 2017; Fairlie et al., 2016; Haussmann et al., 2005). Even though other studies have tested for, but not found such associations (Beaulieu et al., 2011; Sudyka et al., 2014), a meta-analysis in nonhuman vertebrates reported an overall association between short telomeres and higher mortality risk (Wilbourn et al., 2018). While this provides evidence for a link between telomere length and life-history, whether telomere length plays a

direct causal role in senescence, because telomeres are integral to organismal function, or acts as a noncausal biomarker of somatic integrity remains currently unclear (Simons, 2015; Young, 2018).

Compelling evidence exists that early-life conditions such as maternal effects, developmental stress and competition for resources (e.g. Asghar et al., 2015a; Cram et al., 2017; Hausmann et al., 2012) can be particularly influential in shaping telomere length. The greater strength of early-life than late-life effects could be due to stronger forces of selection, since natural selection acts on the proportion of a cohort that is alive, which is greatest in early-life (Hamilton, 1966). However, greater selection in early-life is affected by a trade-off between parental and offspring survival (Lee, 2003; Lee, 2008), causing the evolutionary paradigm around early-life telomere length to remain relatively poorly understood (Vedder et al., 2017). Nevertheless, early-life telomere length might be an important predictor of life-histories (Monaghan, 2010; Wilbourn et al., 2018; Young, 2018). While studies into the effects of the environment on telomeres are emerging in wild mammals (Cram et al., 2017; Izzo et al., 2011; Lewin et al., 2015), longitudinal studies in wild mammals remain relatively rare (Beirne et al., 2014; Fairlie et al., 2016). Gaining a better understanding of telomere dynamics, its relationship with survival, and early-life effects requires more comprehensive longitudinal studies in wild populations.

The European badger (*Meles meles*; henceforth 'badger') provides an informative mammalian model species for studying the effects of early-life conditions on telomere length and senescence patterns. We benefit here from a long-term study of badgers at Wytham Woods (Oxford, UK; Macdonald et al., 2015); an almost closed population (see Macdonald et al., 2008) with a high and relatively consistent annual recapture rate of 84% (SE = 1.3%; Macdonald et al., 2009) over 1726 life-histories monitored seasonally since 1987. In this population, badgers live

in polygynandrous social groups (mean group size: 11.3, range: 2–29; da Silva et al., 1994), and show reproductive senescence (Dugdale et al., 2011b). Badgers have one litter per year (mean litter size  $1.4 \pm 0.06$  SE; range 1–4; Dugdale et al., 2007), where cubs emerge from underground dens at 6–8 weeks of age, are weaned at 12 weeks, and reach independence at 14–16 weeks old (Fell et al., 2006). Cub survival probability ranges from 61–94% (mean  $\pm$  SE =  $67\% \pm 3\%$ ; Macdonald et al., 2009), and cub cohorts are negatively impacted by early-life exposure to endo-parasitic coccidia infection (Newman et al., 2001), oxidative stress (Bilham et al., 2018) and unseasonable weather variation (Macdonald et al., 2010; Noonan et al., 2014; Nouvellet et al., 2013). We therefore posit that strong selection pressures on badger cubs may be reflected in their telomere length and survival probability.

Here, we investigate longitudinal telomere dynamics among 24 cohorts in wild badgers. Relative leukocyte telomere length (RLTL) measurements were used to test: (i) age-related variation in RLTL and the extent to which this was driven by within-individual changes, and both cohort and sex effects; (ii) the repeatability of RLTL and whether within-individual changes in telomere length are attributed to measurement error; and (iii) whether early-life and adult RLTL predict survival and lifespan.

## **2.3 Methods**

### **2.3.1 Study system**

We conducted this study in Wytham Woods, Oxfordshire, UK ( $51^{\circ}46'24''$ N,  $1^{\circ}20'04''$ W), a 424 ha mixed semi-natural woodland site surrounded by mixed arable and permanent pasture (Macdonald and Newman, 2002; Macdonald et al., 2004b; Savill, 2010). The resident high-density badger population (range = 20.5–49.5 badgers/km<sup>2</sup>; Macdonald et al., 2015) forms large social groups (Johnson et al., 2000). Badger social groups have clearly demarcated territories (Buesching et al., 2016; Delahay et al., 2000), although badgers do cross these borders when

foraging and meet amicably with neighbouring groups (Ellwood et al., 2017; Noonan et al., 2015). Mean annual adult survival rates in this population are 0.83 ( $\pm 0.01$  SE; Macdonald et al., 2009) with a mean adult lifespan of 4.96 years ( $\pm 3.21$  SD; Bright Ross, J., Pers. Comm.).

Trapping has been undertaken three or four times per year since 1987, for two to three consecutive days per social group. Trapped badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren et al., 2005) and identified by a unique tattoo number on the left inguinal region. Capture date, sett, social group (comprising several setts, i.e. burrow systems), sex, age-class (cub <1 year old; adult  $\geq 1$  year old) and morphometric measurements (i.e. length, weight, tooth wear; da Silva and Macdonald, 1989; Macdonald et al., 2009) were recorded for each badger. Badger age was defined as the number of days elapsed since the 14<sup>th</sup> of February in their respective birth year (reflecting the February birth peak; Yamaguchi et al., 2006). Blood was collected by jugular venipuncture into vacutainers with an EDTA anticoagulant, and stored at -20°C immediately. Badgers were released at their setts, after full recovery from anaesthesia.

### **2.3.2 Telomere analyses**

We selected 1248 blood samples from 612 individuals, representing 308 males and 304 females, comprising individuals varying in lifespan (range: 14–233 months; mean $\pm$ SE = 97.2 $\pm$ 1.88 months) and from different cohorts ( $n = 24$ ). Only badgers for which age could be determined, either trapped as a cub ( $n = 545$ ) or inferred through low tooth wear, were included ( $n = 67$ ; males = 26, females = 41; tooth wear 1 indicates a cub and tooth wear 2 indicates a 1-year-old adult (da Silva and Macdonald, 1989; Macdonald et al., 2009), where young individuals also had to have length <685 mm and weight <8 kg). Individuals were either sampled once ( $n = 163$ ) or more ( $n = 449$  badgers; 2–9 times per individual) for telomere length analyses. Only badgers which were

considered dead at the time of analysis were included. All analyses were also run without the 67 individuals for which age was determined through tooth wear, to confirm that inclusion of these samples did not bias the results (see supporting results S1).

Genomic DNA was extracted from whole blood using the DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol, with adjustments using 125 µl of anticoagulated blood and a double elution step (2x 75 µl AE buffer). DNA integrity was assessed by running a random selection of DNA extracts (ca. 20%) on agarose gels to check for high molecular weight. DNA concentration of all samples was quantified using the Fluostar Optima fluorometer (BMG Labtech, Ortenberg, Germany) and standardized to 20 ng/µl, after which samples were stored at -20 °C.

Relative leukocyte telomere length (RLTL) measurements were made using the monochrome multiplex qPCR method described by Cawthon (2009). This method provides a ratio of the abundance of telomeric sequence to that of the control gene, the T/S ratio, analysed in the same well which should reduce measurement error by excluding pipetting errors and well effects. DNA samples were assayed using SYBR® Select Master Mix (Applied Biosystems, Warrington, UK) with telomere primers telg (5'-ACA-CTA-AGG-TTT-GGG-TTT-GGG-TTT-GGG-TTT-GGG-TTA-GTG-T-3') and telc (5'-TGT-TAG-GTA-TCC-CTA-TCC-CTA-TCC-CTA-TCC-CTA-TCC-CTA-ACA-3') at a concentration of 900 nM. A GC-clamp was added to the control gene (interphotoreceptor retinoid-binding protein; IRBP) primers to allow for sufficiently different melt temperatures between the control gene and telomeric sequences, using GC-clamped IRBP primers IRBP-F (5'-CGG-CGG-CGG-GCG-GCG-CGG-GCT-GGG-CGG-GCC-ACA-TTT-CTG-GTA-TCC-CCT-3') and IRBP-R (5'-GCC-CGG-CCC-GCC-GCG-CCC-GTC-CCG-CCG-GGG-CGG-TCG-TAG-ATG-GTA-TC-3') at a concentration of 900 nM. Subsequent melt-curve analysis confirmed differential melt-curves and lack of primer-dimer formation. Semi-skirted 96-well polypropylene qPCR plates

were loaded manually with initial reaction volumes of 20 µl. Each well contained 10 µl of SYBR® Select Master Mix (Applied Biosystems, Warrington, UK), 4.9 µl of nuclease free water, 0.9 µM of both the forward and reverse primers (900 nM) and 1.5 µl of 20 ng/µl DNA sample (which was replaced with 1.5 µl of nuclease free water in controls) and sealed with PCR-plate film adhesive. Cycling conditions in the Quantstudio 12K flex real-time PCR system (Applied Biosystems, Warrington, UK) were: 50°C for 2 min and 95°C for 2 min, followed by 2 cycles at 94°C for 15 sec and 49°C for 15 sec, then 40 cycles at 94°C for 15 sec, at 60°C for 10 sec, at 74°C for 15 sec, at 84°C for 10 sec and 86°C for 15 sec. A serially diluted (4x from 80 to 0.3125 ng/µl) ‘reference’ sample was included on each qPCR plate to produce a standard curve to calculate plate efficiencies, where the 20 ng/µl dilution was used as a calibrator. The reference sample was collected from a badger in 2005 and was subject to the same capture methods and long-term storage as the other samples that we analysed.

Samples were randomly allocated to qPCR plates and run in duplicate in adjacent wells, after which amplicon lengths and telomeric sequences were confirmed on the Agilent TapeStation 4200 and 3730 DNA Analyzer (Applied Biosystems, Warrington, UK) with the Big Dye 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK). Cq-values on the 34 qPCR plates declined in a log-linear fashion ( $r^2 > 0.99$ ). Using LinRegPCR 2017.1 (Ruijter et al., 2009) we corrected for baseline fluorescence, determined the windows of linearity for the amplification curves (0.432 for IRBP and 0.694 for telomeres) and calculated efficiencies and Cq-values for each well. Reaction efficiencies were (mean±SE) 1.793±0.004 for IRBP and 1.909±0.004 for telomeres, and we calculated RLTL according to Pfaffl (2001):

$$RLTL = \frac{(E_{tel}^{(Cq_{tel(calibrator)} - Cq_{tel(sample)})})}{(E_{IRBP}^{(Cq_{IRBP(calibrator)} - Cq_{IRBP(sample)})})}$$



where  $E_{tel}$  and  $E_{IRBP}$  represent the mean well efficiencies for each of the amplicons,  $Cq_{tel(calibrator)}$  and  $Cq_{IRBP(calibrator)}$  are the mean Cq-values for the calibrators (20 ng/ $\mu$ l) for each amplicon and  $Cq_{tel(sample)}$  and  $Cq_{IRBP(sample)}$  are the mean Cq-values for both amplicons in each sample.

Inter-plate repeatability (intraclass correlation coefficient), calculated with rptR 0.9.2 (Stoffel et al., 2017), was calculated with the reference sample by comparing variance among duplicates of the reference sample within a plate, to variance of the reference sample among plates and estimated at 0.82 (95% CI = 0.76–0.87;  $n$  = 142 samples; 34 plates). Intra-plate repeatability was calculated with duplicates of the same sample on the same plate, while controlling for plate effects, and estimated at 0.90 (95% CI = 0.86–0.93;  $n$  = 1248 samples; 34 plates) for IRBP, 0.84 (95% CI = 0.79–0.90;  $n$  = 1248 samples; 34 plates) for telomere Cq-values and 0.87 (95% CI = 0.82–0.91;  $n$  = 1248 samples; 34 plates) for RLTL measurements (for further details on quality control see supporting methods).

### 2.3.3 Statistical analyses

Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2019), with RLTL measurements square-root transformed to meet the assumptions of Gaussian error distributions in models with RLTL as the response variable.

#### 2.3.3.1 Age, sex and cohort effects on telomere length

We assessed the relationship between RLTL and age (months), and the interaction with cohort, following Fairlie et al. (2016) and Spurgin et al. (2017). We tested a variety of age functions in General Linear Mixed Models (GLMMs; Bates et al., 2015) that included individual ID, plate ID and year as random effects, and sex, sample storage time (months), and in some models cohort, as fixed effects. We checked for collinearity and found that sample storage time and cohort were

collinear ( $VIF > 3$ ), since sample storage time is similar within cohorts. We therefore first determined that sample storage time was not associated with telomere length ( $\beta = -0.006 \pm 0.010$  SE,  $X^2 = 0.383$ , d.f. = 1,  $P = 0.536$ ) and then excluded it from subsequent models. We considered a null model (without the age terms), polynomial age terms (linear, quadratic, cubic), a full-factorial age term and a variety of threshold functions. Visual inspection of the data indicated inflection points, with further specification of inflection points through comparison of AIC values, at 29, 65 and 112 months of age. These threshold models (with either a single, double or triple threshold) were compared to all other models. We ran additional models to test whether adding a cohort fixed effect and an interaction between age and cohort improved the model, using AIC values. We did not fully apply model selection or averaging, as we aimed to compare a set of specifically defined models, where the model with the lowest AIC fits these data best, but we considered all plausible models with  $\Delta AIC < 7$ .

We then tested age-specific sex differences in telomere length through an interaction between age and sex in the best fitting age model and all non-significant interactions were dropped. In the same model we included age at last capture ( $\alpha_i$ ), as a measure of lifespan (van de Pol and Verhulst, 2006), to test if selective disappearance of individuals contributed to the age pattern observed. We also compared, in the same model, within-individual ( $\beta_w$ ) to between-individual ( $\beta_B$ ) slopes, where the difference between these slopes is exactly the effect of selective disappearance (van de Pol and Verhulst, 2006). In a separate model we tested the significance of the between-individual component by replacing age parameters by within-group deviation scores (age -  $\alpha_i$ ).

### 2.3.3.2 Individual repeatability and telomere elongation

Individual repeatability (across multiple samples from the same individual) was calculated by dividing the variance explained by individual identity by total phenotypic variance, in a Gaussian-distributed model (identity link function), across all samples ( $n = 1248$ ) and then only for adult samples ( $n = 779$ ). These models included RLTL as the response variable and the best fitting age variable and cohort as fixed effects, with individual ID and plate ID as random effects. The variance explained by plate ID was then excluded from the total phenotypic variance as it is a source of experimental measurement error and therefore not biologically relevant phenotypic variance; thus, it could lead to underestimation of repeatability (Dochtermann et al., 2015). Additionally, we determined the correlation between within-individual telomere measurements, using the marginal  $R^2$  (Nakagawa and Schielzeth, 2013), in a Gaussian-distributed model (identity link function) with RLTL as the response variable, RLTL at  $t+1$ , cohort and age (months) as fixed effects and individual ID as a random effect.

We examined increases in RLTL with age by estimating differences in telomere lengths among technical replicates, i.e. duplicates next to each other within a plate, and among within-individual samples, i.e. difference in RLTL between within-individual samples. We used MCMCglmm (Hadfield, 2010) with an inverse Wishart prior ( $v = 1$ ,  $\nu = 0.002$ ), 600,000 iterations, a thinning of 300 and burn-in period of 15,000 iterations, to test whether within-individual changes in RLTL were greater than measurement error. We randomly selected two samples per individual, and built a model with RLTL as the response variable and individual ID and plate ID as random effects ( $n = 898$  samples; 449 individuals). We then randomly selected one set of duplicates per individual, and constructed a model with RLTL for each of the technical replicates as the response variable and individual ID as a random effect ( $n = 898$  samples; 449 individuals). We compared the explained variance by the random effect for individual ID between these two models and whether the 95% credible intervals overlapped. Additionally, we separated the

dataset into groups that either increased or decreased in RLTL and ran these models again for these groups separately. We also tested if the residual error variance ( $\bar{\sigma}_{\varepsilon}^2$ ) was smaller than the error variance in RLTL, when RLTL can increase or decrease ( $\sigma_{\varepsilon'}^2$ ), following Simons et al. (2014), which would reject the hypothesis that RLTL shows no elongation.

#### 2.3.3.3 Telomere length, survival and lifespan

We used GLMMs to test the relationship between early-life RLTL (<1 year old) and lifespan ( $n = 435$ ). In the following models, we conducted model averaging, using an information theoretic approach to select plausible models and estimate the relative importance of fixed effects for models with  $\Delta AIC < 7$  with the “natural average method” (Burnham et al., 2011). All four models included sex as a fixed factor, and plate ID and natal social group as random effects. Early-life RLTL did not vary with age ( $n = 435$ ,  $\beta = -0.002 \pm 0.006$  SE,  $X^2 = 0.160$ , d.f. = 1,  $P = 0.690$ ); therefore, age was not included in GLMMs with early-life RLTL as a fixed effect. Firstly, early-life RLTL as a predictor of lifespan was modelled with lifespan as the response variable ( $n = 435$ ), including early-life RLTL and cohort as additional fixed effects in a Poisson-distributed model (log link function). We also controlled for overdispersion by including observation (for each unique measure) as a random effect (Harrison, 2014). Lifespan was determined as the age at last capture. To ensure the different survival probabilities for cubs and adults did not alter the results we also ran a model (see Table S2.1) with lifespan calculated in months as the difference between the date of birth and last capture, with 24 months added when last captured as adults, due to a 95% recapture interval of 2 years (Dugdale et al., 2007), and 12 months as cub, due to their different survival rates (Macdonald et al., 2009). Secondly, we modelled survival to adulthood ( $\geq 1$  year old) using a binary term in a binomial (logit link function) mixed-effects model with early-life RLTL ( $n = 435$ ) and cohort as additional fixed effects. Thirdly, we used a Cox mixed-

effects model to test whether early-life RLTL predicts annual adult survival probability over the lifetime of individuals that survived their first year. The model included early-life RLTL ( $n = 336$ ) as an additional fixed effect, and cohort as an additional random effect. Finally, we tested the relationship between adult RLTL ( $n = 779$ ) and survival to the subsequent year, in a binomially-distributed model (logit link function) with RLTL interacting with age (based on the best fitting model) as an additional fixed effect and individual ID (correcting for multiple measures per individual), cohort, current social group and year as additional random effects.

## **2.4 Results**

### **2.4.1 Age, sex and cohort effects on telomere length**

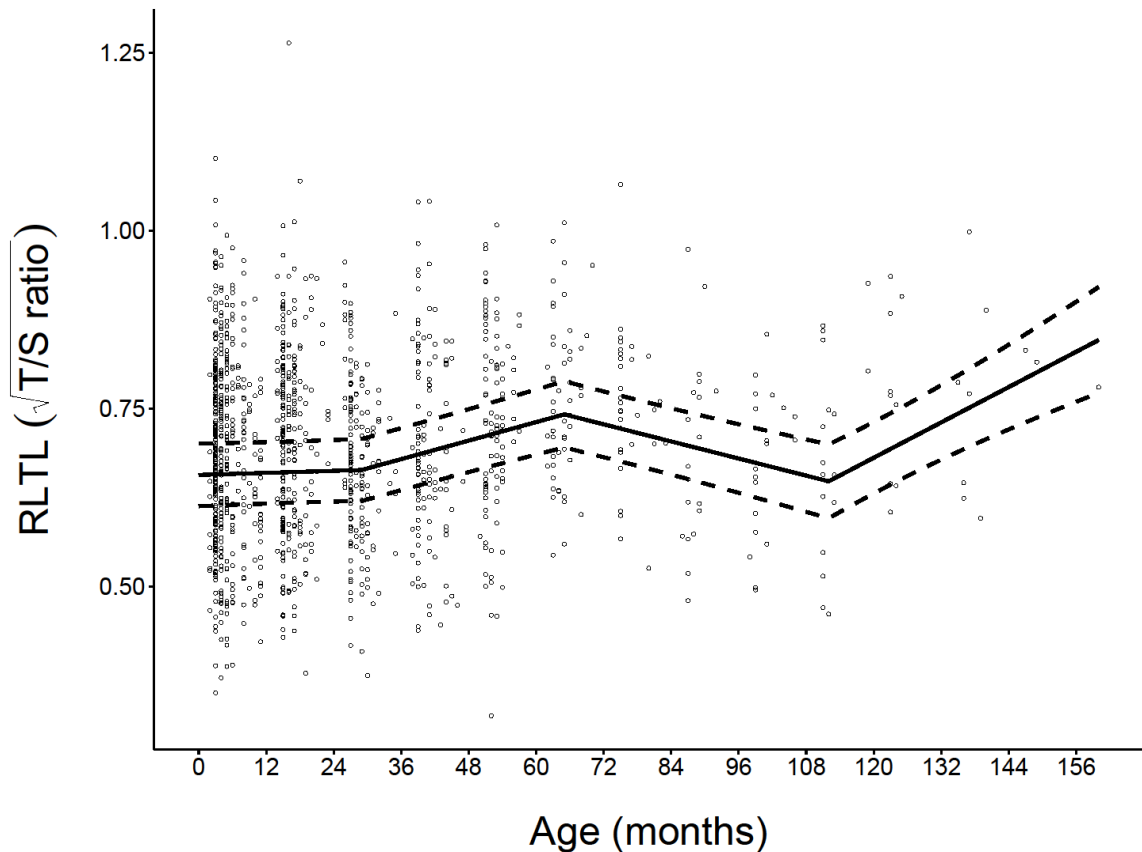
Across all samples, after no change up to and including 29 months of age, RLTL increased up to and including 65 months, followed by a decline up to and including 112 months, with a second increase in RLTL in older age (Table 2.1; Figure 2.1). Two models had  $\Delta AIC < 7$ , with the top model including all thresholds, and the second-best model with thresholds at 65 and 112 months, where both models included a fixed factor for cohort (Table S2.2 and Figure S2.1). Males and females had similar telomere lengths (Table 2.1) and there was no evidence for different age patterns by sex. Cohorts from earlier years (1987–1992) had lower and more variable early-life RLTL measurements than those from subsequent years (Figure 2.2a). We thus repeated these analyses where these cohorts were omitted, which showed that these cohorts did not alter the results (see supporting results S2).

**Table 2.1: Parameter estimates from the models that best explained the relationship between telomere length and age, when accounting for selective disappearance ( $n = 1248$  samples; 612 individuals).  $\beta_w$  = within-individual slope,  $\beta_s$  = selective disappearance according to age at last capture,  $\beta_B$  = between-individual slope,  $\alpha_i$  = between-individual component, S.E. = standard error, d.f. = degrees of freedom.  $P$ -values from log-likelihood ratio tests, where significant parameters are in bold.**

Parameters	$\beta$	S.E.	d.f.	p-value	$\beta_B (\beta_s + \beta_w)$
Model 1 <sup>†</sup> :					
Intercept	0.6259	0.0527			
Age ( $\leq 29$ months) ( $\beta_w$ )	0.000029	0.00054	1	0.958	0.000199
<b>(&gt;29, <math>\leq 65</math> months) (<math>\beta_w</math>)</b>	<b>0.002130</b>	<b>0.00051</b>	<b>1</b>	<b>&lt;0.001</b>	<b>0.002301</b>
<b>(&gt;65, <math>\leq 112</math> months) (<math>\beta_w</math>)</b>	<b>-0.00210</b>	<b>0.00063</b>	<b>1</b>	<b>&lt;0.001</b>	<b>-0.001924</b>
<b>(&gt; 112 months) (<math>\beta_w</math>)</b>	<b>0.004008</b>	<b>0.00143</b>	<b>1</b>	<b>0.005</b>	<b>0.004179</b>
Sex (male)	0.008045	0.00687	1	0.242	
<b>Cohort<sup>§</sup></b>			<b>23</b>	<b>&lt;0.001</b>	
Lifespan ( $\beta_s$ )	0.000171	0.000093	1	0.068	
Model 2 <sup>†</sup> :					
Intercept	0.6259	0.0527			
Age ( $\leq 29$ months) ( $\beta_w$ )	0.000029	0.00054	1	0.958	
<b>(&gt;29, <math>\leq 65</math> months) (<math>\beta_w</math>)</b>	<b>0.002130</b>	<b>0.00051</b>	<b>1</b>	<b>&lt;0.001</b>	
<b>(&gt;65, <math>\leq 112</math> months) (<math>\beta_w</math>)</b>	<b>-0.00210</b>	<b>0.00063</b>	<b>1</b>	<b>&lt;0.001</b>	
<b>(&gt; 112 months) (<math>\beta_w</math>)</b>	<b>0.004008</b>	<b>0.00143</b>	<b>1</b>	<b>0.005</b>	
Sex (male)	0.008045	0.00687	1	0.242	
<b>Cohort<sup>§</sup></b>			<b>23</b>	<b>&lt;0.001</b>	
<b><math>\alpha_i (\beta_B)</math></b>	<b>0.004242</b>	<b>0.00138</b>	<b>1</b>	<b>0.004</b>	

Random effect estimates (variance): <sup>†</sup>Individual ID ( $4.851 \times 10^{-5}$ ), Plate ( $1.067 \times 10^{-3}$ ), Social group ( $6.062 \times 10^{-5}$ ), Year ( $3.731 \times 10^{-3}$ ), Residual ( $1.295 \times 10^{-2}$ ); <sup>§</sup>Estimates  $\pm$  S.E. for 24 cohorts are in the supporting information (Figure S2.1).

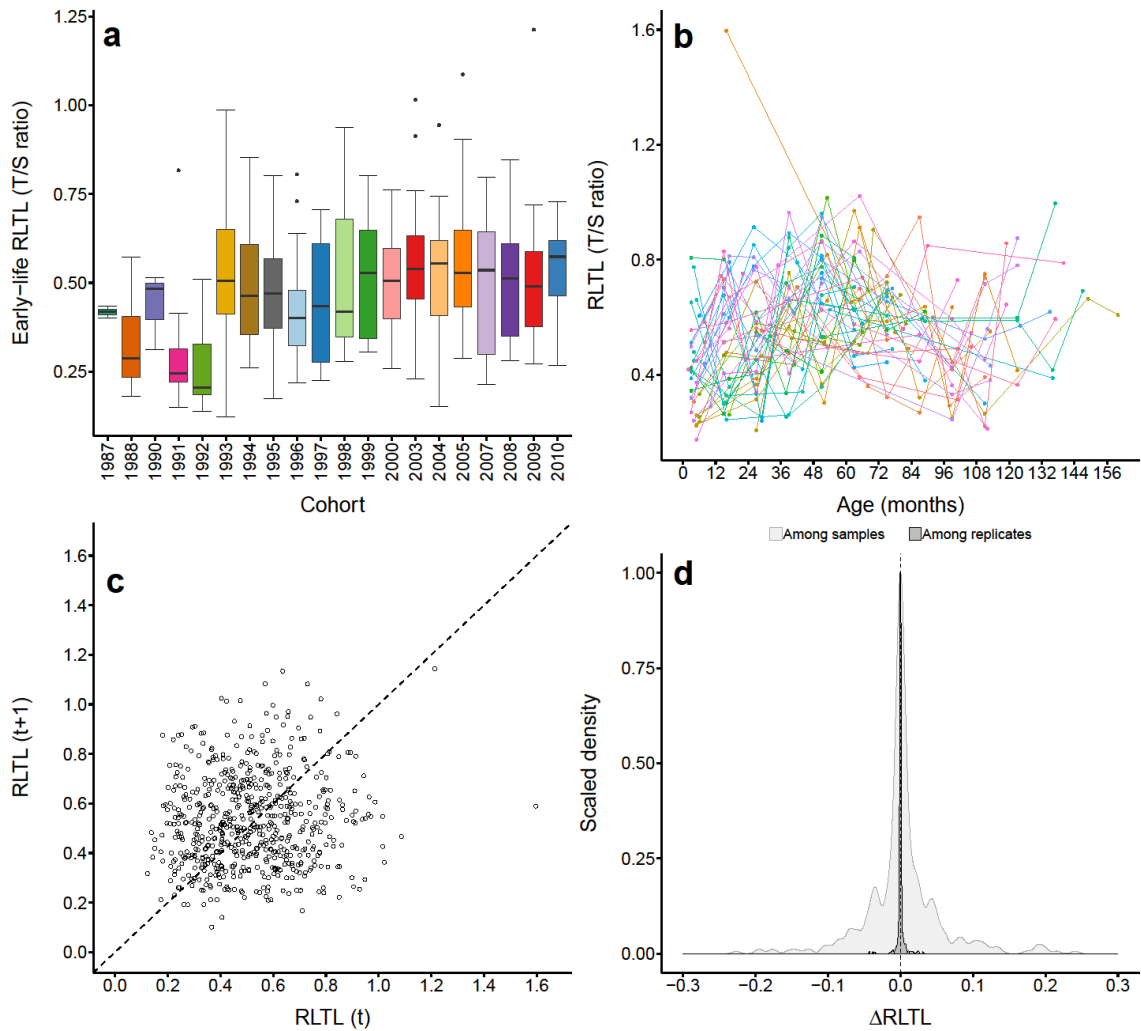
Selective disappearance of individuals was accounted for by including age at last capture ( $\beta_s$ ) in the best fitting age model, which was borderline significant (Table 2.1). However, there was a between-individual effect ( $\beta_B$ ) and a within-individual effect ( $\beta_w$ ) for individuals aged 29 months or older, where the difference between these slopes is due to selective disappearance of individuals with shorter telomeres (Table 2.1). Consequently, selective disappearance of individuals with shorter telomeres did contribute to the age pattern observed.



**Figure 2.1: Age-related variation in relative leukocyte telomere length (RLTL), with inflection points at 29, 65 and 112 months of age. Raw data points ( $n = 1,248$ ) are shown with fitted lines representing the model prediction for RLTL (T/S ratio) with 95% confidence intervals.**

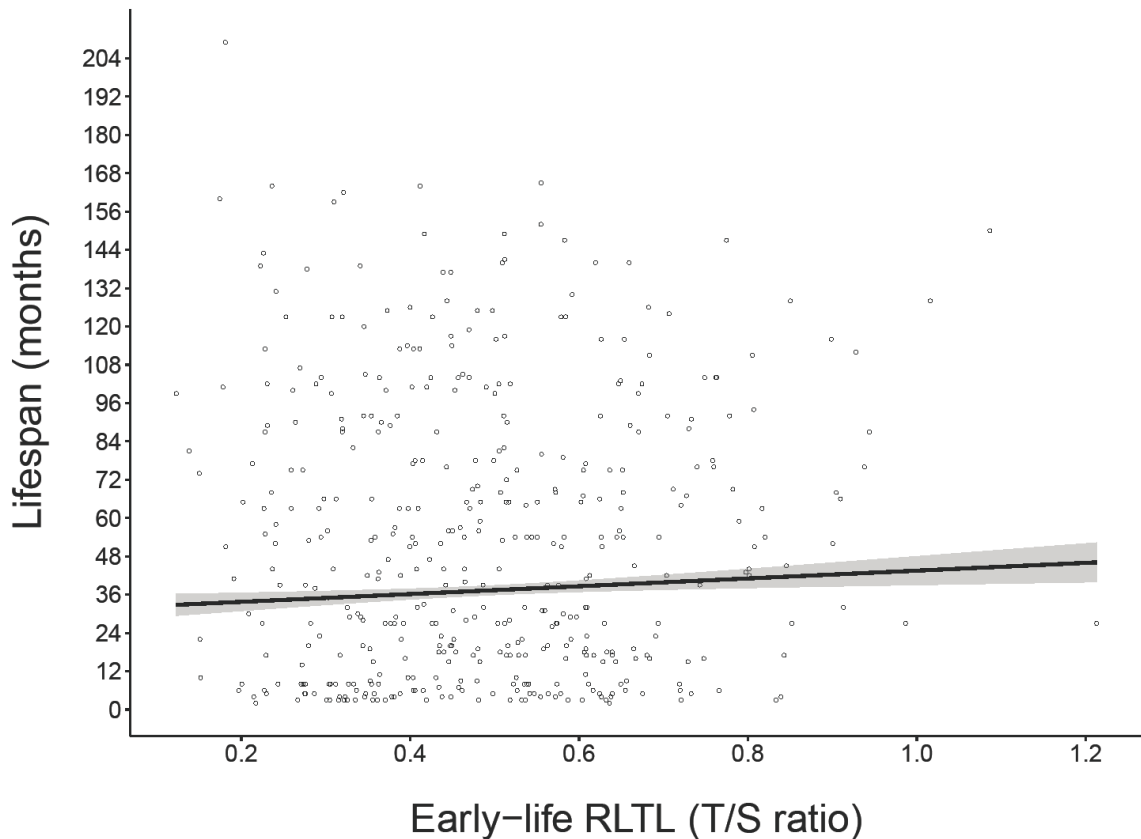
#### **2.4.2 Individual repeatability and telomere elongation**

Individual repeatability was 0.017 (95% CI = 0.001–0.098) including cub and adult RLTL estimates, and 0.026 (95% CI = 0.001–0.143) using only RLTL measurements from adulthood. These repeatabilities changed to 0.022 (95% CI = 0.001–0.103) and 0.039 (95% CI = 0.001–0.154), respectively, when plate variance (measurement error) was removed from the phenotypic variances, so 2.2% of the variance in RLTL was explained by within-individual consistency among samples. There was no significant correlation between RLTL measured at different time points in the same individual (marginal  $R^2 = 0.067$ ;  $X^2 = 0.92$ ,  $P = 0.336$ ; Figure 2.2b).



**Figure 2.2: Telomere dynamics in European badgers. a) Variation in early-life relative leukocyte telomere length (RLTL) among cohorts. b) Longitudinal telomere dynamics for 41 individuals that were measured at least four times. c) Within-individual variation in RLTL over consecutive time points (t and t+1). Dashed line represents parity, thus data points above and below this line represent increases and decreases in telomere length, respectively. d) Scaled density plots of changes in RLTL among technical replicates (dark grey) and among individual samples (light grey) with a dotted line representing no change. Areas left of the dotted line represent decreases in RLTL, while to the right represent increases.**

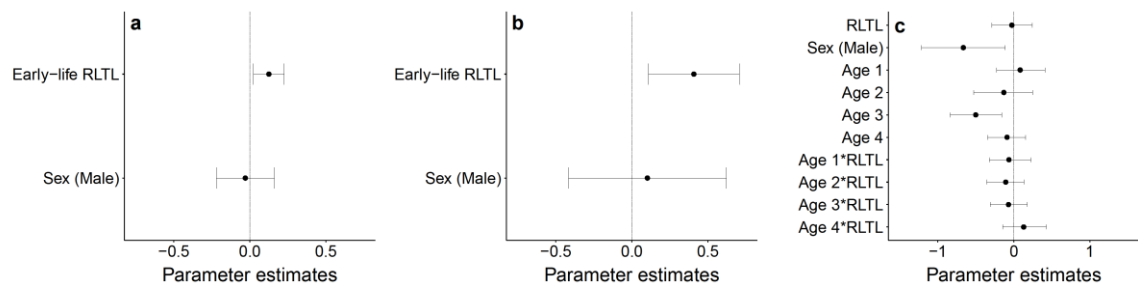




**Figure 2.3: Early-life (<1 year old) relative leukocyte telomere length (RLTL) predicts lifespan. Raw data ( $n = 435$ ) are shown as open circles, the regression from the GLMM as a black line, and the 95% confidence interval as the shaded area.**

Increases (in the range of 0.004–5.829% per month) in RLTL were identified in 61.2% of within-individual changes (Figure 2.2c) for individuals with  $\geq 2$  samples ( $n = 449$ ). When accounting for plate effects using MCMCglmm, the random effect estimate for individual ID with technical replicates was 0.0331 (95% CI = 0.0290–0.0376), whereas for within-individual samples the random effect estimate was 0.0014 (95% CI = 0.0003–0.0044; Figure 2.2d). For the group that exhibited increases in RLTL the random effect estimate for individual ID with technical replicates was 0.0345 (95% CI = 0.0289–0.0424), whereas for within-individual samples this estimate was 0.0016 (95% CI = 0.0003–0.0058). The random effect estimate for technical replicates in the group that exhibited decreases in RLTL was 0.0359 (95% CI = 0.0310–0.0452)

and for within-individual samples this estimate was 0.0006 (95% CI = 0.0003–0.0045), where none of the 95% credible intervals from the technical replicates and within-individual samples overlapped. Additionally, residual variance among samples was smaller ( $\bar{\sigma}_{\varepsilon}^2 = 0.041$ ) than the overall change in RLTL ( $\sigma_{\varepsilon'}^2 = 0.922$ ;  $F_{31,40} = 22.48$ ,  $P < 0.001$ ). These within-individual increases in RLTL were therefore not solely due to measurement error.

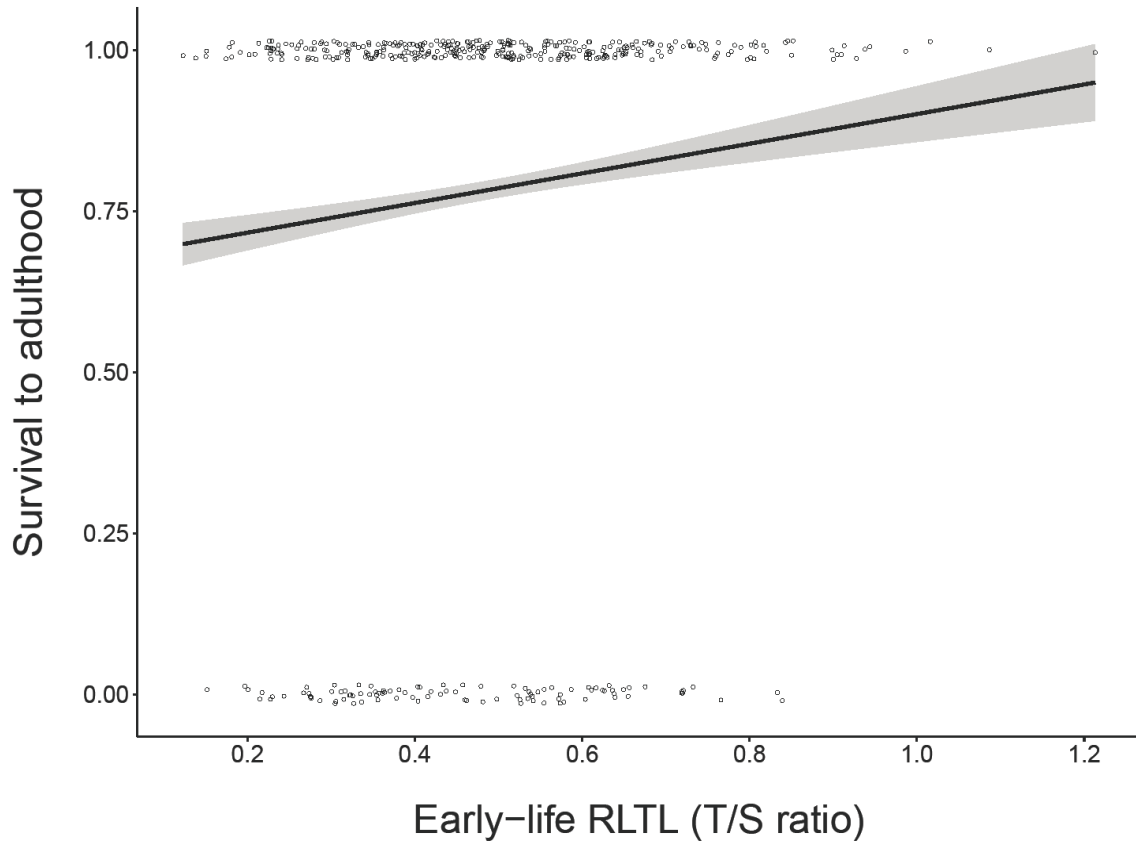


**Figure 2.4: Parameter estimates and 95% confidence intervals of fixed effects from models investigating the effect of: a) Early-life RLTL (relative leukocyte telomere length) on lifespan; b) Early-life RLTL on survival to adulthood; and, c) Adult RLTL on survival to the next year. Age parameters in plot c) refer to threshold model where Age 1  $\leq 29$  months old, Age 2  $> 29$  and  $\leq 65$  months old, Age 3  $> 65$  and  $\leq 112$  months old and Age 4  $> 112$  months old. Scale differs in plot c). For cohort effects see Figure S2.2. \* represents an interaction.**

### 2.4.3 Telomere length, survival and lifespan

Early-life RLTL (<1 year old) was positively associated with lifespan (Figure 2.3 and 2.4a; Table S2.3 and S2.4), where individuals with longer telomeres in early-life had longer lifespans, such that an increase of 1 T/S ratio was associated with 13.3% greater longevity. However, this association was underpinned by survival benefits in early-life and not in adulthood as early-life RLTL only predicted survival to adulthood (Figure 2.5 and 2.4b; Table S2.4 and S2.5). In contrast, early-life RLTL showed no relationship with annual adult survival probability (Table S2.4) and adult RLTL showed no association with survival to the subsequent year (Figure 2.4c; Table S2.4

and S2.6), but all models indicated an effect of cohort on survival and lifespan (Figure S2.2; Table S2.4).



**Figure 2.5: Early-life (<1 year old) relative leukocyte telomere length (RLTL) predicts survival to adulthood ( $\geq 1$  year old). The regression line from a binomial GLMM is shown, with associated 95% confidence interval as a shaded area, and raw jittered data as open circles ( $n = 435$ ).**

## 2.5 Discussion

We found complex telomere dynamics with no apparent change ( $\leq 29$  months of age), decreases (i.e. between  $>65$  and  $\leq 112$  months) and increases in RLTL with age ( $>29$  and  $\leq 65$ , and  $>112$  months). This pattern was mainly due to within-individual changes. However, selective disappearance of individuals with shorter telomeres contributed to the age pattern observed when age at last capture was included (as a measure of selective disappearance) and within- and

between-individual slopes were compared. While the lack of change in RLTL in early-life contrasts with previous studies that have reported rapid declines in RLTL with age in early-life (Aubert and Lansdorp, 2008; Baerlocher et al., 2003), we are unable to sample individuals until at least 3 months of age, due to welfare legislation (Protection of Badgers Act, 1992), and therefore we may miss the period where the greatest changes in RLTL occur. The combination of selective mortality and within-individual changes in RLTL was also reported in wild Soay sheep (*Ovis aries*; Fairlie et al., 2016), providing evidence for complex relationships between telomere length and age.

Male and female badgers had similar telomere lengths across all ages, corroborating recent findings in wild meerkats (*Suricata suricatta*) and European badgers in Woodchester (Beirne et al., 2014; Cram et al., 2017), but contrasting with age-specific sex differences in telomere length in Soay sheep (*Ovis aries*; Watson et al., 2017). The lack of age-specific sex differences in badgers and meerkats could be due to males and females having similar lifespans, whereas in Soay sheep females live much longer than males (Cram et al., 2017; Fairlie et al., 2016; Macdonald and Newman, 2002).

Individual repeatability in RLTL was only 2.2% throughout an individual's lifespan. The point estimate was higher (3.9%) when only including RLTL measurements in adulthood, but the 95% confidence intervals overlapped greatly, and within-individual RLTL measurements were not correlated. Within-individual RLTL correlations in humans were high (0.82–0.93; Benetos et al., 2013) and individual repeatability in RLTL in avian TRF studies was also high (81%–83%; Bauch et al., 2013; Boonekamp et al., 2014). In contrast, lifelong qPCR studies in wild populations provide substantially lower repeatability estimates (7%; Spurgin et al., 2017; 13%; Fairlie et al., 2016). The individual repeatability estimate in RLTL in our system is in the lower spectrum of qPCR-studies. Such a low individual repeatability indicates that the within-individual slopes in

RLTL across ages are different. RLTL is therefore highly variable within individuals across their lifetimes, where positive within-individual changes indicate some active process in increasing telomere length.

Telomere elongation, particularly in qPCR-based studies, is often attributed to measurement error (Steenstrup et al., 2013; Verhulst et al., 2015). It is, however, becoming more apparent in wild population studies that telomeres do elongate (Fairlie et al., 2016; Hoelzl et al., 2016a; Hoelzl et al., 2016b; Kotrschal et al., 2007; Spurgin et al., 2017). Our study supports this, using monochrome multiplex qPCR that, in principle, reduces measurement error due to reactions occurring in the same well. Additionally, we found that residual variance among samples was smaller than the overall change in RLTL, and variance among technical replicates was smaller than among-sample variation, indicating that increases in mean telomere length with age were not due to measurement error alone.

Aside from actual telomere elongation, however, we acknowledge the potential for competing mechanisms that could alter mean RLTL, notably changes in leukocyte cell composition with age (Kimura et al., 2010; Linton and Dorshkind, 2004; Pawelec et al., 2010; Weng, 2012). Mammalian leukocytes are nucleated and different leukocyte cell types have different telomere lengths due to their respective functional capacities to proliferate and express telomerase (Aubert and Lansdorp, 2008; Weng, 2001), and these vary in ratio over time with health/immune status (see Davis et al., 2008). For instance, an innate immune response can cause a granulocyte-biased leukocyte ratio, where in humans and baboons the granulocytes have longer telomeres than lymphocytes (Baerlocher et al., 2007; Kimura et al., 2010). While a previous study of RLTL in wild Soay sheep did not find changes in leukocyte cell composition with age (Watson et al., 2017), leukocyte cell composition in badgers does vary between similar aged cubs and across an individual's lifespan due to changes in immune system activation (Montes,

2007). A greater metabolic rate while clearing infection could also modify leukocyte cell composition and potentially affect mean RLTL directly. For instance, badger cubs are typically infected with coccidia (Newman et al., 2001), causing a strong innate immune response and oxidative stress (Bilham et al., 2013; Bilham et al., 2018). A change in an individual's immunological status, along with age, may therefore alter individual leukocyte cell composition and might contribute to RLTL elongation in this study.

Our study shows a positive relationship between early-life RLTL and lifespan, driven by survival benefits of long telomeres in early-life, rather than in adulthood. This is congruent with previous studies reporting that early-life RLTL predicts lifespan more strongly than RLTL in adulthood (Fairlie et al., 2016; Heidinger et al., 2012) and where early-life RLTL predicts survival to adulthood in nonhuman mammals (Cram et al., 2017; Fairlie et al., 2016). Early-life RLTL in badgers does predict survival to adulthood, but not adult survival probability. Cubs have higher mortality rates than adults (Macdonald et al., 2009), which could drive this association between early-life RLTL and lifespan. In contrast, adult RLTL in badgers did not predict survival to the following year, whereas other studies found that adult RLTL does predict survival to the next year (e.g. Barrett et al., 2013). The lack of such an association in our study system could be due to, for example, most of our RLTL measurements in later adulthood ( $\geq 2$  years) being from long-lived individuals, indicating a sampling bias with fewer samples in later adulthood from individuals with shorter lifespans. The interplay between adult RLTL and the adult environment, or in combination with the early-life environment, also requires understanding to explain the link between adult RLTL and adult survival to the next year. Even though early-life RLTL predicts survival probability in badgers, it remains currently unclear how RLTL and life-history are linked (Simons, 2015; Young, 2018). A direct link might exist through delayed cellular senescence when telomeres are longer (von Zglinicki et al., 2001). However, an indirect link exists when telomeres

function as a biomarker of somatic redundancy and reflect the accumulated damage to other biological structures that have deleterious effects on fitness (Boonekamp et al., 2013; Young, 2018).

The early-life environment clearly exerted a strong effect on early-life RLTL, apparent from the pronounced variation in early-life RLTL we noted among cohorts, which corroborates the variation in survival rate and lifespan among cohorts in our study system (Macdonald and Newman, 2002; Macdonald et al., 2010). Badgers in our study are exposed to variable environmental conditions and have a limited tolerance for, for example, cohort-specific weather conditions (i.e. higher cub recruitment and survival probability with intermediate levels of rainfall and restricted deviation from the mean temperature; Nouvellet et al., 2013; Macdonald et al., 2010) and exposure to diseases (i.e. lower cub survival probability with higher intensities of coccidia; Newman et al., 2001). These variable environmental conditions may be reflected in the variation in early-life telomere length seen in our study system. Similarly, previous studies in birds have shown that higher levels of early-life competition can accelerate telomere shortening (Boonekamp et al., 2014; Nettle et al., 2015), although studies that do not find stressors affecting early-life telomere length do exist (reviewed in Vedder et al., 2017). In mammals, studies on social and ecological effects on telomere dynamics are emerging (Cram et al., 2017; Izzo et al., 2011; Lewin et al., 2015; Watson et al., 2017; Wilbourn et al., 2017), showing that, for example, socially dominant spotted hyaenas (*Crocuta crocuta*) have longer telomeres (Lewin et al., 2015) and that meerkat pups experiencing more intense early-life competition have shorter telomeres (Cram et al., 2017).

As well as environmental effects, variation in early-life RLTL can also be caused by additive genetic effects (Dugdale and Richardson, 2018). In wild populations, using a quantitative genetic 'animal model', no heritability of telomere length was found in white-throated dippers

(*Cinclus cinclus*; Becker et al., 2015), and high heritability (0.35–0.48) was found in the great reed warbler (*Acrocephalus arundinaceus*; Asghar et al., 2015a). Even though we currently have no heritability estimates from wild mammals, the likelihood for additive genetic effects in our study system to contribute to early-life RLTL is small given that individual repeatability, which sets the upper limit for heritability (unless indirect genetic effects occur), in RLTL is low. This indicates that the individual variation in RLTL in our study system is likely driven by early-life environmental conditions.

Our findings demonstrate that telomeres reflect the effects of early-life conditions on individual life-history, and elaborate on the dynamic way that telomeres function as a biomarker of senescence in a wild mammal, where within-individual telomere length is highly variable. Further work on how specific early-life environment conditions impact telomere lengths in wild mammals and quantifying the relative contribution of environmental effects (e.g. cohort, year and social group) on telomere length will provide insight into the evolution of senescence.



# Supplemental information Chapter 2: Individual variation in early-life telomere length and survival in a wild mammal

## Supplementary methods

### *Quality control of telomere length estimation through quantitative PCR*

LinRegPCR 2017.1 (Ruijter et al., 2009) was used to correct for baseline fluorescence, to determine the window of linearity for each amplicon (i.e. separate windows for IRBP and telomere reactions) and to calculate amplification efficiencies for each well. Subsequently, Cq-values for each sample were calculated in R 3.3.1 (R Development Core Team, 2019). Across plates ( $n = 34$ ), fluorescence thresholds ( $N_q$ ) were set to a constant value within the window of linearity for the amplification curves: 0.432 for IRBP and 0.694 for telomeres. Mean amplification efficiency across wells for each amplicon group per plate, excluding outliers (outside the 5<sup>th</sup> and 95<sup>th</sup> percentiles), were used as our estimates of reaction efficiency (as recommended by Ruijter et al., 2009).

Further quality control was applied, where samples were excluded from further analyses if the standard deviation across their duplicate Cq values for either amplicon group was greater than 5% of the mean Cq for that sample ( $n = 25$ ). We also excluded any sample if the standard deviation across the duplicate well-specific efficiencies for either amplicon was greater than 5% of the overall mean efficiency for that amplicon group ( $n = 44$ ). Lastly, samples with a Cq-value >28 for telomere, or >29 for IRBP, were excluded from the analysis ( $n = 24$ ), assuming that these were failed reactions. In order to determine failed reactions for control samples, we applied a similar rule where samples with a standard deviation of the duplicate T/S ratios >8% of the mean

T/S ratio for that sample were excluded, as at least one of the duplicate samples was assumed to have failed ( $n = 19$ ; <12% of samples).

Reaction efficiencies differed between our IRBP and telomere reactions (mean efficiencies across all samples on all plates run: IRBP =  $1.793 \pm 0.004$  SE; Telomere:  $1.909 \pm 0.004$  SE). Assuming constant amplification efficiencies across plates can bias qPCR results when these actually differ, we therefore calculated relative leukocyte telomere length (RLTL) using a method that does not assume consistent efficiencies across plates (Pfaffl, 2001):

$$RLTL = \frac{(E_{tel}^{(Cq_{tel(calibrator)} - Cq_{tel(sample)})})}{(E_{IRBP}^{(Cq_{IRBP(calibrator)} - Cq_{IRBP(sample)})})}$$

In this equation,  $E_{tel}$  and  $E_{IRBP}$  represent the mean well efficiencies for each of the amplicons, calculated in LinRegPCR,  $Cq_{tel(calibrator)}$  and  $Cq_{IRBP(calibrator)}$  are the mean Cq-values for the calibrators (20 ng/ $\mu$ l) of the reference sample for each amplicon and  $Cq_{tel(sample)}$  and  $Cq_{IRBP(sample)}$  are the mean Cq-values for both amplicons in each sample.

Only 69 of 1324 samples did not pass the initial quality control and these samples were repeated. 17 repeated samples passed the quality control, meaning that 52 samples (<4%) were excluded. Additionally, as 24 samples had a Cq-value >28 for TL or Cq-value >29 for IRBP, which we considered to be failed reactions, these were excluded from the analyses. This resulted in a total of 1248 RLTL measurements from 612 individuals (308 males and 304 females), with 163 individuals having 1 sample (early-life samples to reduce bias from viability selection), 408 individuals with 2 samples, 5 individuals with 5 samples, 17 individuals with 6 samples, 12 individuals with 7 samples, 5 individuals with 8 samples and 2 individuals with 9 samples.

Inter-plate repeatability (intraclass correlation coefficient), calculated with rptR 0.9.2 (Stoffel et al., 2017), was 0.82 (95% CI = 0.76–0.87). Intra-plate repeatability was 0.90 (95% CI = 0.86–0.93) and 0.84 (95% CI = 0.79–0.90) for IRBP and telomere Cq values, respectively, with an intra-plate repeatability of 0.87 (95% CI = 0.82–0.91;  $n = 1248$  samples; 34 plates) for RLTL measurements.

### **Results S1 - summary of analyses excluding individuals aged based on tooth wear**

The following summary results represent the exact same analyses, but without the 67 individuals whose age was determined based on tooth wear.

Individual repeatability was 0.014 (95% CI = 0.001–0.100) including cub and adult RLTL and 0.026 (95% CI = 0.001–0.158) with only RLTL measurements in adulthood (marginal  $R^2 = 0.072$ ;  $\chi^2 = 0.930$ ,  $P = 0.335$ ). These repeatabilities changed to 0.020 (95% CI = 0.001–0.101) and 0.032 (95% CI = 0.001–0.156), respectively, when plate variance was removed. This did not differ from the estimates including the 67 individuals (0.017, 95% CI = 0.001–0.098, all samples; 0.026, 95% CI = 0.001–0.143, adult samples).

Telomere elongation was observed in 60.9% of within-individual changes (versus 61.2% including the 67 individuals). The random effect estimate for individual ID with technical replicates was 0.0369 (95% CI = 0.0320–0.0429), whereas for within-individual samples the random effect estimate was 0.0009 (95% CI = 0.0003–0.0045) without overlapping 95% credible intervals. For the group that increased in RLTL, the random effect estimate for technical replicates was 0.0417 (95% CI = 0.0345–0.0516) and for within-individual samples the estimate was 0.0012 (95% CI = 0.0002–0.0054). For individuals that exhibited decreases in RLTL the random effect estimate for technical replicates was 0.0392 (95% CI = 0.0317–0.0470) and for

within-individual samples the estimate was 0.0014 (95% CI = 0.0003–0.0065), where neither group showed overlapping 95% credible intervals. Residual variance ( $\sigma_{\epsilon}^{-2}$ ) was smaller (0.041) than the overall change ( $\sigma_{\epsilon}^{\prime 2}$ ) in RLTL (0.922;  $F_{31,40} = 22.48$ ,  $P < 0.001$ ). Removing the 67 individuals therefore did not alter our conclusions on telomere elongation.

Removing the 67 individuals also did not alter our conclusions on the relationship between RLTL and survival/lifespan: Early-life RLTL predicted lifespan ( $\beta = 0.132$ , 95% CI = 0.028–0.236;  $n = 424$ ), while early-life RLTL also showed a positive relationship with survival to adulthood ( $\beta = 0.434$ , 95% CI = 0.132–0.734;  $n = 424$ ). Early-life RLTL ( $\beta = 0.043$ , SE = 0.350,  $P = 0.900$ ;  $n = 325$ ) and corresponding RLTL ( $\beta = -0.028$ , 95% CI = -0.329–0.276;  $n = 663$ ) did not predict adult survival probability.

The addition of individuals aged using tooth wear did therefore not alter our results or conclusions.

## **Results S2 – summary of analyses when first cohorts (1987 – 1992) are omitted**

The following summary results represent the exact same analyses, but without the six (1987–1992) cohorts where early-life RLTL was lower and more variable than other cohorts.

Individual repeatability was 0.030 (95% CI = 0.001–0.123; including all samples) and 0.052 (95% CI = 0.001–0.206) with only RLTL measurements in adulthood, (marginal  $R^2 = 0.060$ ;  $\chi^2 = 1.501$ ,  $P = 0.220$ ). These repeatabilities changed to 0.035 (95% CI = 0.001–0.126) and 0.063 (95% CI = 0.001–0.220), respectively, when plate variance was removed.

Telomere elongation was observed in 59.2% of within-individual changes. The random effect estimate for individual ID with technical replicates was 0.0340 (95% CI = 0.0288–0.0388), whereas for within-individual samples the random effect estimate was 0.0018 (95% CI = 0.0002–0.0044) without overlapping 95% credible intervals. For the group that increased in RLTL, the

random effect estimate for technical replicates was 0.0410 (95% CI = 0.0328–0.0515) and for within-individual samples the estimate was 0.0008 (95% CI = 0.0003–0.0055). For individuals that exhibited decreases in RLTL the random effect estimate for technical replicates was 0.0315 (95% CI = 0.0264–0.0413) and for within-individual samples the estimate was 0.0012 (95% CI = 0.0002–0.0059), where neither group without overlapping 95% credible intervals. Residual variance ( $\sigma_{\varepsilon}^{-2}$ ) was smaller (0.038) than the overall change ( $\sigma_{\varepsilon}'^2$ ) in RLTL (0.872;  $F_{20,27} = 23.01$ ,  $P < 0.001$ ).

Early-life RLTL predicted lifespan ( $\beta = 0.119$ , 95% CI = 0.019–0.219;  $n = 389$ ), while early-life RLTL also showed a positive relationship with survival to adulthood ( $\beta = 0.430$ , 95% CI = 0.118–0.742;  $n = 389$ ). Early-life RLTL ( $\beta = -0.125$ , SE = 0.369,  $P = 0.730$ ;  $n = 299$ ) and corresponding RLTL did not predict adult survival probability ( $\beta = -0.070$ , 95% CI = -0.363–0.221;  $n = 594$ ).

The addition of individuals from cohorts 1987–1992 did not alter any results or conclusions.

**Table S2.1: The different survival probabilities of cubs and adults, based on age at last capture and when correcting the lifespan model for the different survival probabilities between cubs and adults (+12 months last captured as cubs, +24 months for adults). Model averaged parameters of models.  $\Sigma$  = relative variable importance,  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval; with reference terms in brackets = reference level for factors. Parameters where the 95% CI do not overlap zero are italicised and underlined.**

<b>Parameter (reference level)</b>	<b><math>\Sigma</math></b>	<b><math>\beta</math></b>	<b>S.E.</b>	<b>95% CI</b>
<b>Lifespan (corrected for differential survival probability)</b>				
Intercept		4.059	0.469	3.141 to 4.984
<i>Early-life RLTL</i>	<i><u>0.86</u></i>	<i><u>0.084</u></i>	<i><u>0.034</u></i>	<i><u>0.016 to 0.152</u></i>
Sex (female)	0.27	-0.031	0.065	-0.158 to 0.097
<i>Cohort</i>	<i><u>1.00</u></i>			
<b>Lifespan (based on age of last capture)</b>				
Intercept		3.531	0.702	2.158 to 4.916
<i>Early-life RLTL</i>	<i><u>0.86</u></i>	<i><u>0.125</u></i>	<i><u>0.052</u></i>	<i><u>0.024 to 0.227</u></i>
Sex (female)	0.25	-0.030	0.097	-0.220 to 0.161
<i>Cohort</i>	<i><u>1.00</u></i>			

**Table S2.2: Comparison of models describing the relationship between relative leukocyte telomere length and age, with a variety of age functions (0 = no age function, 1 = linear age function, 2 = log age function, 3 = quadratic age function, 4 = cubic age function, F = factorial age function, T1 = single threshold, T2 = double threshold, T3 = triple threshold) and cohort, including the interaction of cohort with age (cohort \* age), with plate, year and individual ID as random effects and sex as a fixed effect. Models were ordered and numbered by AICc, and the difference from the top model (lowest AICc) is stated in the column termed ‘ $\Delta$ AICc’, with models within  $\Delta$ AICc <7 in bold.**

Model	Function	Thresh hold 1	Thresh hold 2	Thresh hold 3	Cohort	Cohort*Age	Degrees of freedom	AICc	$\Delta$ AICc
1	T3	29	65	112	Yes	No	34	<b>-1704.412</b>	
2	T2	65	112		Yes	No	33	<b>-1701.832</b>	<b>2.580</b>
3	T2	29	65		Yes	No	33	-1695.173	9.239
4	T1	65			Yes	No	32	-1694.749	9.663
5	1				Yes	No	31	-1691.114	13.298
6	3				Yes	No	32	-1690.745	13.667
7	2				Yes	No	31	-1689.680	14.732
8	T1	112			Yes	No	32	-1689.574	14.838
9	T1	29			Yes	No	32	-1689.344	15.068
10	4				Yes	No	33	-1689.256	15.156
11	T3	29	65	112	No	No	11	-1688.943	15.469
12	T2	29	112		Yes	No	33	-1688.306	16.106
13	0				Yes	No	30	-1684.658	19.754
14	T2	65	112		No	No	10	-1682.605	21.807
15	T2	29	65		No	No	10	-1681.564	22.848
16	1				No	No	8	-1679.276	25.136
17	T1	112			No	No	9	-1679.217	25.195
18	T1	29			No	No	9	-1678.154	26.258
19	T1	65			No	No	9	-1678.106	26.306
20	0				No	No	7	-1678.082	26.330
21	2				No	No	8	-1677.796	26.616
22	T2	29	112		No	No	10	-1677.428	26.984
23	3				No	No	9	-1677.272	27.140
24	4				No	No	10	-1675.252	29.160
25	2				Yes	Yes	54	-1663.010	41.402
26	1				Yes	Yes	54	-1658.236	46.176
27	T2	65	112		Yes	Yes	74	-1656.381	48.031
28	T1	112			Yes	Yes	62	-1655.049	49.363
29	T1	65			Yes	Yes	67	-1649.490	54.922
30	T1	29			Yes	Yes	75	-1639.517	64.895
31	T3	29	65	112	Yes	Yes	95	-1635.637	68.775
32	T2	29	112		Yes	Yes	83	-1633.420	70.992
33	3				Yes	Yes	78	-1632.109	72.303
34	T2	29	65		Yes	Yes	88	-1630.728	73.684
35	4				Yes	Yes	102	-1623.823	80.589
36	F				Yes	No	118	-1620.230	84.182
37	F				No	No	95	-1609.840	94.572
38	F				Yes	Yes	380	-1219.151	485.261

**Table S2.3: Model selection of factors linked to lifespan. Models retained for subsequent model averaging are in bold. ✓ = categorical term included in the model, Int = intercept ( $\pm$ SE), RLTL = relative leukocyte telomere length ( $\pm$ SE), d.f. = degrees of freedom,  $\Delta$ AICc = change in Akaike Information Criterion (AICc) relative to best supported model,  $\omega$  = adjusted weight based on AICc.**

<b>Model</b>	<b>Int</b>	<b>RLTL</b>	<b>Sex</b>	<b>Cohort</b>	<b>d.f.</b>	<b><math>\Delta</math>AICc</b>	<b><math>\omega</math></b>
<b>1</b>	<b>3.534 <math>\pm</math> 0.701</b>	<b>0.126 <math>\pm</math> 0.052</b>		✓	<b>24</b>	<b>0.00</b>	<b>0.641</b>
<b>2</b>	<b>3.547 <math>\pm</math> 0.703</b>	<b>0.125 <math>\pm</math> 0.052</b>	✓	✓	<b>25</b>	<b>2.16</b>	<b>0.218</b>
<b>3</b>	<b>3.491 <math>\pm</math> 0.706</b>			✓	<b>23</b>	<b>3.63</b>	<b>0.105</b>
<b>4</b>	<b>3.506 <math>\pm</math> 0.707</b>		✓	✓	<b>24</b>	<b>5.75</b>	<b>0.036</b>
5	3.499 $\pm$ 0.088				4	43.02	0.000
6	3.497 $\pm$ 0.088	0.062 $\pm$ 0.053			5	43.68	0.000
7	3.522 $\pm$ 0.104		✓		5	44.91	0.000
8	3.519 $\pm$ 0.105	0.062 $\pm$ 0.053	✓		6	45.59	0.000



**Table S2.4: Model averaged parameters.  $\Sigma$  = relative variable importance,  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95 % CI = 95 % confidence interval; reference terms in brackets = reference level; for cohort effect estimates see Figure S2.2. \* = interaction. Parameters where the 95% CI do not overlap zero are italicised and underlined.**

Parameter (reference level)	$\Sigma$	$\beta$	S.E.	95% CI
<b>Lifespan model<sup>†</sup></b>				
Intercept		3.531	0.702	2.158 to 4.916
<i>Early-life RLTL</i>	<i>0.86</i>	<i>0.125</i>	<i>0.052</i>	<i>0.024 to 0.227</i>
Sex (female)	0.25	-0.030	0.097	-0.220 to 0.161
<i>Cohort</i>	1.00			
<b>Survival to adulthood model<sup>‡</sup></b>				
Intercept		0.053	1.478	-2.847 to 2.965
<i>Early-life RLTL</i>	<i>0.95</i>	<i>0.408</i>	<i>0.153</i>	<i>0.108 to 0.708</i>
Sex (female)	0.25	0.102	0.264	-0.417 to 0.622
<i>Cohort</i>	1.00			
Parameter (reference level)		$\beta$	S.E.	P-value
<b>Annual adult survival model<sup>§</sup></b>				
Early-life RLTL		0.032	0.346	0.930
<i>Sex (female)</i>		<i>0.352</i>	<i>0.127</i>	<i>0.006</i>
Parameter (reference level)	$\Sigma$	$\beta$	S.E.	95% CI
<b>Adult survival to following year model<sup>¶</sup></b>				
Intercept		2.325	0.466	1.400 to 3.235
Adult RLTL	0.37	-0.026	0.135	-0.290 to 0.242
<i>Sex (female)</i>	<i>0.94</i>	<i>-0.664</i>	<i>0.280</i>	<i>-1.214 to -0.114</i>
Age ( $\leq 29$ months)	0.28	0.084	0.160	-0.227 to 0.411
(>29 and $\leq 65$ months)	0.31	-0.131	0.191	-0.528 to 0.246
<i>(&gt;65 and <math>\leq 112</math> months)</i>	<i>0.99</i>	<i>-0.501</i>	<i>0.171</i>	<i>-0.836 to -0.158</i>
(> 112 months)	0.32	-0.089	0.125	-0.344 to 0.156
Age ( $\leq 29$ months)*RLTL)	0.01	-0.064	0.129	-0.320 to 0.225
(>29 and $\leq 65$ months)*RLTL)	0.03	-0.107	0.091	-0.357 to 0.133
(>65 and $\leq 112$ months)*RLTL)	0.08	-0.070	0.115	-0.310 to 0.176
(> 112 months)*RLTL)	0.04	0.130	0.143	-0.145 to 0.428

Model averaged random effect estimates (variance): <sup>†</sup>Observation (0.8730), Plate (4.330\*10<sup>-15</sup>), Natal social group (0.1751); <sup>‡</sup>Plate (0.000), Natal social group (0.3193); <sup>§</sup>Cox mixed model random effect estimates: Cohort (0.2816), Plate (0.0481), Natal social group (0.3963); <sup>¶</sup>Individual ID (0.4620), Cohort (0.1415), Year (0.8757), Plate (0.2894), Natal social group (1.919\*10<sup>-6</sup>), Current social group (1.2300).

Table S2.5: Model selection of factors linked to survival to adulthood. Models retained for subsequent model averaging are in bold. ✓ = categorical term included in the model, Int = intercept ( $\pm$ SE), RLTL = relative leukocyte telomere length ( $\pm$ SE), d.f. = degrees of freedom,  $\Delta$ AICc = change in Akaike Information Criterion (AICc) relative to best supported model,  $\omega$  = adjusted weight based on AICc.

Model	Int	RLTL	Sex	Cohort	d.f.	$\Delta$ AICc	$\omega$
<b>1</b>	<b>0.072 <math>\pm</math> 1.476</b>	<b>0.407 <math>\pm</math> 0.153</b>		✓	<b>23</b>	<b>0.00</b>	<b>0.694</b>
<b>2</b>	<b>0.022 <math>\pm</math> 1.484</b>	<b>0.410 <math>\pm</math> 0.153</b>	✓	✓	<b>24</b>	<b>2.09</b>	<b>0.244</b>
<b>3</b>	<b>-0.064 <math>\pm</math> 1.471</b>			✓	<b>22</b>	<b>5.42</b>	<b>0.046</b>
4	-0.099 $\pm$ 1.478		✓	✓	23	7.57	0.016
5	1.271 $\pm$ 0.140	0.254 $\pm$ 0.122			4	16.18	0.000
6	1.201 $\pm$ 0.185	0.256 $\pm$ 0.122	✓		5	17.89	0.000
7	1.254 $\pm$ 0.138				3	18.62	0.000
8	1.189 $\pm$ 0.184		✓		4	20.37	0.000

Table S2.6: Model selection of factors linked to adult survival probability. Models retained for subsequent model averaging are in bold.  $\checkmark$  = categorical term included in the model, \* interaction between two terms, Int = intercept ( $\pm$ SE), RLTL = relative leukocyte telomere length ( $\pm$ SE), Age parameters ( $\pm$ SE) refer to threshold model where Age 1  $\leq$  29 months old, Age 2  $>$  29 months and  $\leq$  65 months old, Age 3  $>$  65 months and  $\leq$  112 months old and Age 4  $>$  112 months old, d.f. = degrees of freedom,  $\Delta$ AICc = change in Akaike Information Criterion (AIC) relative to best supported model,  $\omega$  = adjusted weight based on AICc. Table only includes models with  $\Delta$ AICc  $\leq$  7 (not showing 159 models).

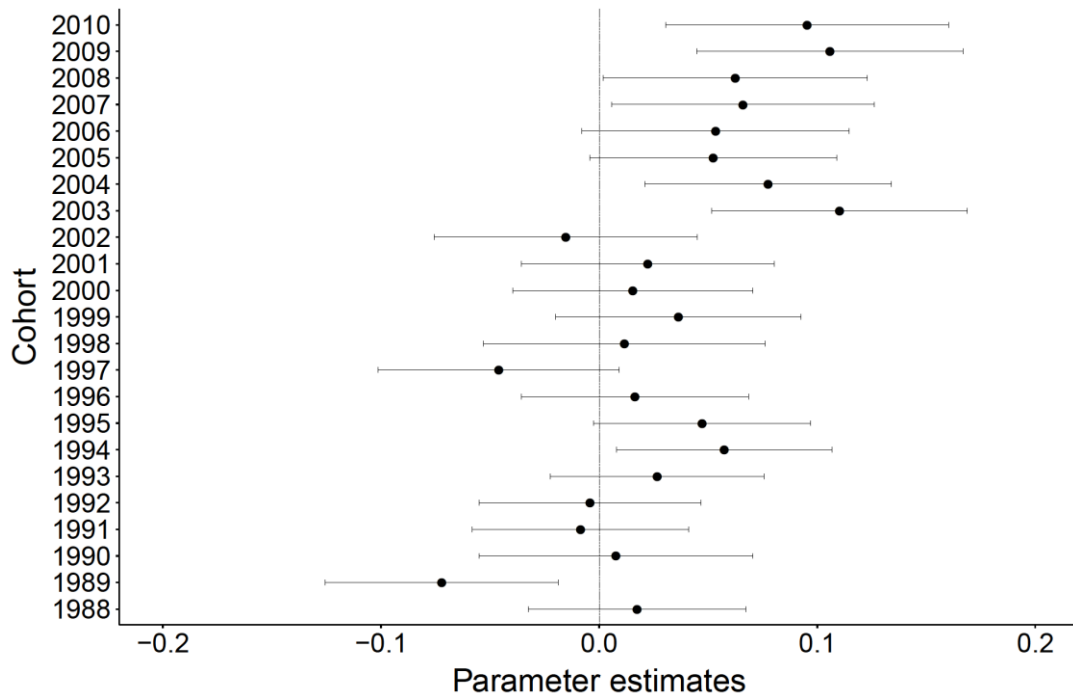
Model	Int	RLTL	Age 1	Age 2	Age 3	Age 4	Sex	Age 1* RLTL	Age 2* RLTL	Age 3* RLTL	Age 4* RLTL	d.f.	$\Delta$ AICc	$\omega$
1	<b>2.342</b> $\pm$ <b>0.461</b>				<b>-0.532</b> $\pm$ <b>0.157</b>		$\checkmark$					9	0.00	0.166
2	<b>2.362</b> $\pm$ <b>0.465</b>				<b>-0.488</b> $\pm$ <b>0.170</b>	<b>-0.078</b> $\pm$ <b>0.119</b>	$\checkmark$					10	1.62	0.074
3	<b>2.348</b> $\pm$ <b>0.464</b>			<b>-0.097</b> $\pm$ <b>0.176</b>	<b>-0.489</b> $\pm$ <b>0.175</b>		$\checkmark$					10	1.75	0.069
4	<b>2.338</b> $\pm$ <b>0.459</b>		<b>0.053</b> $\pm$ <b>0.143</b>		<b>-0.537</b> $\pm$ <b>0.157</b>		$\checkmark$					10	1.92	0.064
5	<b>2.348</b> $\pm$ <b>0.464</b>	<b>-0.034</b> $\pm$ <b>0.136</b>			<b>-0.537</b> $\pm$ <b>0.160</b>		$\checkmark$					10	1.99	0.061
6	<b>2.341</b> $\pm$ <b>0.459</b>		<b>0.141</b> $\pm$ <b>0.174</b>	<b>-0.188</b> $\pm$ <b>0.209</b>	<b>-0.459</b> $\pm$ <b>0.176</b>		$\checkmark$					11	3.15	0.034
7	<b>2.371</b> $\pm$ <b>0.469</b>			<b>-0.114</b> $\pm$ <b>0.178</b>	<b>-0.432</b> $\pm$ <b>0.191</b>	<b>-0.088</b> $\pm$ <b>0.121</b>	$\checkmark$					11	3.27	0.032
8	<b>2.358</b> $\pm$ <b>0.462</b>		<b>0.049</b> $\pm$ <b>0.143</b>		<b>-0.494</b> $\pm$ <b>0.170</b>	<b>-0.076</b> $\pm$ <b>0.119</b>	$\checkmark$					11	3.56	0.028
9	<b>2.366</b> $\pm$ <b>0.462</b>	<b>-0.026</b> $\pm$ <b>0.143</b>			<b>-0.493</b> $\pm$ <b>0.170</b>	<b>-0.076</b> $\pm$ <b>0.119</b>	$\checkmark$					11	3.64	0.027

	±	±		±	±														
	0.467	0.137		0.173	0.120														
10	2.362	-0.023		-0.531		✓													
	±	±		±															
	0.465	0.137		0.160															
11	2.354	-0.030		-0.094	-0.494	✓													
	±	±		±	±														
	0.467	0.137		0.177	0.178														
12	2.344	-0.032	0.052		-0.542	✓													
	±	±	±		±														
	0.461	0.135	0.143		0.159														
13	2.003				-0.504														
	±				±														
	0.413				0.153														
14	2.364		0.146	-0.208	-0.399	-0.091	✓												
	±		±	±	±	±													
	0.463		0.174	0.210	0.192	0.120													
15	2.349	-0.032			-0.488	-0.132	✓												
	±	±			±	±													
	0.465	0.137			0.173	0.138													
16	2.345	-0.020	0.139	-0.185	-0.463		✓												
	±	±	±	±	±														
	0.461	0.136	0.175	0.210	0.179														
17	2.374	-0.019		-0.112	-0.436	-0.087	✓												
	±	±		±	±	±													
	0.470	0.138		0.179	0.195	0.122													
18	2.368	-0.017		-0.099	-0.485		✓												
	±	±		±	±														
	0.468	0.139		0.177	0.179														
19	2.063	-0.019		-0.085	-0.464		✓												
	±	±		±	±														
	0.001	0.001		0.001	0.001														
20	2.373	-0.019			-0.500	-0.062	✓												
	±	±			±	±													

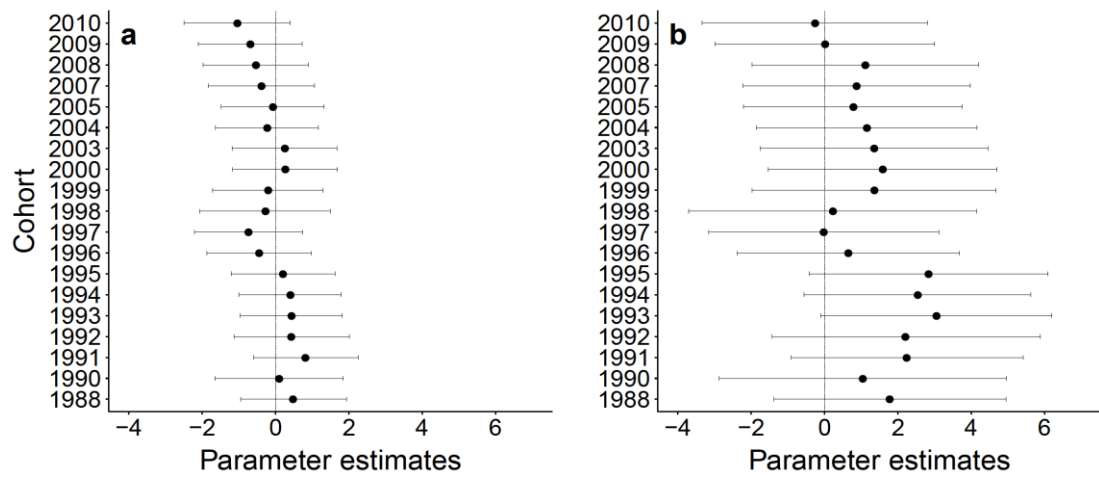
	0.467	0.138			0.174	0.124		0.109				
21	2.362	-0.025	0.048		-0.500	-0.074	✓			12	5.59	0.010
	±	±	±		±	±						
	0.464	0.136	0.143		0.173	0.120						
22	2.358	-0.021	0.054		-0.535		✓	-0.060		12	5.65	0.010
	±	±	±		±			±				
	0.463	0.137	0.143		0.159			0.105				
23	2.352	-0.035	0.052		-0.539		✓	-0.064		12	5.73	0.009
	±	±	±		±			±				
	0.463	0.136	0.143		0.159			0.129				
24	2.009		0.090		-0.514					9	5.79	0.009
	±		±		±							
	0.411		0.137		0.152							
25	2.019				-0.461	-0.076				9	5.79	0.009
	±				±	±						
	0.416				0.167	0.118						
26	2.352	-0.017			-0.487	-0.142	✓	-0.152	0.202	13	5.84	0.009
	±	±			±	±		±	±			
	0.463	0.137			0.173	0.139		0.139	0.157			
27	2.001			-0.062	-0.474					9	6.07	0.008
	±			±	±							
	0.414			0.169	0.173							
28	2.009	-0.041			-0.509					9	6.11	0.008
	±	±			±							
	0.415	0.135			0.156							
29	2.368	-0.007	0.141	-0.172	-0.471		✓	-0.110		13	6.45	0.007
	±	±	±	±	±			±				
	0.465	0.137	0.175	0.211	0.180			0.122				
30	2.356	-0.025		-0.111	-0.431	-0.143	✓		0.104	13	6.68	0.006
	±	±		±	±	±			±			
	0.468	0.138		0.179	0.194	0.139			0.128			
31	2.366	-0.009	0.145	-0.207	-0.401	-0.090	✓			13	6.70	0.006
	±	±	±	±	±	±						
	0.464	0.137	0.175	0.212	0.196	0.121						

32	2.391	-0.009		-0.096	-0.453	-0.072	✓	-0.100		13	6.75	0.006
	±	±		±	±	±		±				
	0.473	0.139		0.181	0.197	0.123		0.126				
33	2.360	-0.006	0.146	-0.194	-0.452		✓	-0.067		13	6.86	0.005
	±	±	±	±	±			±				
	0.462	0.138	0.175	0.211	0.179			0.105				
34	2.264			-0.307		-0.218	✓			10	6.93	0.005
	±			±		±						
	0.426			0.153		0.108						
35	2.345	-0.030	0.045		-0.493	-0.129	✓		0.102	13	6.98	0.005
	±	±	±		±	±			±			
	0.463	0.137	0.143		0.173	0.138			0.127			

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**Figure S2.1: Parameter estimates ( $\pm$ SE) for cohort effects from the best-fitting age model (Table 2.1; main text), with telomere length across all ages as the response variable, relative to 1987.**



**Figure S2.2: Parameter estimates and associated 95% confidence intervals for cohort effects (relative to 1987) in models of: a) Lifespan – early-life RLTL (relative leukocyte telomere length) and b) Survival to adulthood – early-life RLTL mixed models.**



## **Chapter 3: Social effects on age-related and sex-specific immune cell profiles in a wild mammal**

### **3.1 Abstract**

Evidence for age-related changes in innate and adaptive immune responses is increasing in wild populations. Such changes have been linked to fitness, and understanding the factors driving variation in immune responses is important for the evolution of immunity and senescence. Age-related changes in immune profiles may be due to sex-specific behaviour, physiology and responses to environmental conditions. Social conditions may also contribute to variation in immunological responses, for example, through transmission of pathogens and stress from resource and mate competition. Yet, the impact of the social environment on age-related changes in immune cell profile requires further investigation in the wild. Here, we tested the relationship between leukocyte cell composition (agranulocyte proportion, i.e. adaptive and innate immunity) and age, sex, and group size in a wild population of European badgers (*Meles meles*). We found that the proportion of agranulocytes decreased with age only in males living in small groups. In contrast, females in larger groups exhibited a greater age-related decline in the proportion of agranulocytes compared to females in smaller groups. Our results provide evidence for age-related changes in immune cell profiles in a wild mammal, which are influenced by both the sex of the individual and their social environment.

### **3.2 Introduction**

The immune system involves multiple mechanisms that protect the host against pathogens (Hoebe et al., 2004). The functioning of the immune system is related to sex (Restif and Amos, 2010; Klein and Flanagan, 2016) and changes throughout life (Nussey et al., 2012; Cheynel et al., 2017; Peters et al., 2019; Palacios et al., 2011; Schneeberger et al., 2014; Froy et al., 2019). Since age-related changes in immune responses have been linked to mortality in the wild (Froy et al., 2019), understanding the factors driving differences in immune responses can provide insight into the evolution of immunity and senescence.

The immune system comprises two components: innate and adaptive immunity (Hoebe et al., 2004). The innate immune response is the first defence against pathogens,

involving phagocytic cells (e.g. neutrophils, macrophages and dendritic cells) to detect antigens and produce cytokines that trigger other parts of the immune system (Weiskopf et al., 2009; Akira et al., 2006; Nathan, 2006; Mantovani et al., 2011; Vivier et al., 2011). The activation of adaptive immunity includes the cell-mediated immune response, with the stimulation of T lymphocytes and humoral immunity, which is controlled by activated B lymphocytes that can differentiate to produce immunoglobulins against specific antigens (Mantovani et al., 2011; Iwasaki and Medzhitov, 2010). The relative components of adaptive and innate immunity are therefore reflected in agranulocytes (i.e. lymphocytes and monocytes) and granulocytes (i.e. neutrophils, eosinophils and basophils), respectively (Fest et al., 2018; Hu et al., 2014; Templeton et al., 2014; van der Willik et al., 2019).

The adaptive immune system generally undergoes an age-related decline in performance, i.e. immunosenescence, and evidence for this process has been emerging in wild populations (Nussey et al., 2012; Cheynel et al., 2017; Peters et al., 2019; Palacios et al., 2011; Schneeberger et al., 2014; Froy et al., 2019). In contrast, the innate immune response is usually maintained, or even enhanced with age (Nussey et al., 2012; Cheynel et al., 2017; Peters et al., 2019; Palacios et al., 2011; Schneeberger et al., 2014; Froy et al., 2019). This enhanced innate immune response can be a consequence of overstimulation of the immune system, due to a reduced T cell repertoire and bias towards CD8<sup>+</sup> effector memory cells, leading to chronic inflammation and accelerated immunosenescence, as seen in humans (Sansoni et al., 2014; Franceschi et al., 2018).

The innate and adaptive immune responses, mediated by genes and hormones, are sex-specific (Klein and Flanagan, 2016; Restif and Amos, 2010). For example, in the human innate immune response, males have higher frequencies of natural killer cells and higher phagocytic activity of neutrophils and macrophages than females (Abdullah et al., 2012; Spitzer, 1999), whereas in the adaptive immune response, females have stronger antibody responses and have higher basal immunoglobulin levels and B cell numbers than males

(Abdullah et al., 2012; Furman et al., 2014). Such sex differences in immune responses may be exacerbated with age (Klein and Flanagan, 2016; Campo and Davila, 2002). For example, male Soay sheep (*Ovis aries*) exhibit steeper sex-specific changes in leukocyte cell composition with age (Watson et al., 2017). However, such changes may be species-specific since no sex differences in the rate of change in leukocyte cell composition with age were detected in roe deer (*Capreolus capreolus*; Cheynel et al., 2017).

Social stress is emerging as a potential driver of variation in immune responses in the wild (Côté and Poulin, 1995; Creel et al., 2013; Altizer et al., 2003), where gregarious species often experience greater stress due to social interactions or increased mate competition (Blumstein et al., 2018; Creel et al., 2013; Martin, 2009). For instance, polygynous males have more circulating testosterone than conspecific females or monogamous males, which has a suppressive effect on the immune system (Klein, 2000; Hillgarth and Wingfield, 1997), indicating a potential role for the social system and the environment in sex-specific immune cell profiles. Moreover, social species may experience the costs of increased pathogen exposure due to group-living compared with solitary species (Altizer et al., 2003). For example, greater early-life exposure to pathogen variety and intensity within social groups could prime the immune system and result in enhanced later-life immunity with the risk of late-life auto-immunity (Olszak et al., 2012; von Mutius, 2007). However, to date, there has been no clear evidence for the effects of the social environment on sex-specific immune cell profiles and their age-related changes.

Here, we use blood samples collected across 2017 and 2018 from a wild population of European badgers (*Meles meles*; hereafter 'badger') to explore longitudinal changes in sex-specific immune cell profiles and how this relates to social conditions. We quantify the relative components in the immune system through the proportion of agranulocytes out of the total number of leukocytes, which reflect the relative balance between adaptive and innate immunity (Fest et al., 2018; Hu et al., 2014; Templeton et al., 2014; van der Willik et

al., 2019). Specifically, we test whether the proportion of agranulocytes: (i) changes with age, (ii) exhibits sex differences, and (iii) is linked to group size.

### **3.3 Methods**

#### **3.3.1 Study species and data collection**

We conducted this study in Wytham Woods, Oxfordshire, UK (51°46'24"N, 1°20'04"W), a 424 ha semi-natural woodland surrounded by mixed arable pasture (Macdonald and Newman, 2002). The resident high-density badger population (mean±SE = 36±3 badgers/km<sup>2</sup>; Macdonald et al., 2009) is segregated into large mixed-sex social groups (mean group size = 11, range = 2–29; da Silva et al., 1994). Badgers have a polygynandrous mating system with high extra-group paternity (Dugdale et al., 2007; Annavi et al., 2014b), where males exhibit seasonal peaks in testosterone levels (Buesching et al., 2009; Sugianto et al., 2019b). Badgers are exposed to pathogens such as coccidia which negatively impacts development and causes juvenile mortality (Newman et al., 2001; Anwar et al., 2000; Sin et al., 2014).

Trapping was undertaken three times per year, for three consecutive days per social group. Trapped badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren et al., 2005). Individuals were identified by a unique tattoo number on the left inguinal region, with capture date, social group affiliation and sex recorded. Age was determined as the difference between capture date and the 14<sup>th</sup> of February in the respective birth years. Badgers first caught as adults were aged through tooth wear (van Lieshout et al., 2019), where a tooth wear score of 2 typically indicates a 1-year old adult. Blood was collected through jugular venipuncture into vacutainers with EDTA anticoagulant. Badgers were released at their setts, after full recovery from anaesthesia. Additionally, bait-marking was conducted periodically to delimit social group range sizes (Delahay et al., 2000) and calculate group sizes using appropriate dispersal rules (see supporting information).

Immediately after blood collection, one drop of blood from the vacutainers was smeared on a glass microscope slide. Slides were air-dried for one hour and subsequently stained using a Kwik-Diff staining kit (Thermo Scientific, Manchester, UK) according to the manufacturer's protocol. Leukocyte cell counts were conducted by the same observer (blind to group size and sex) by counting 100 cells per slide (4 repeats per slide, not consecutively to avoid bias;  $n = 82$  slides, 23 individuals; 9 females, 14 males), at 40x magnification using the 'battlement technique' (Schalm, 1965). Cells were identified as neutrophils, eosinophils and basophils (i.e. granulocytes) or lymphocytes and monocytes (i.e. agranulocytes; Bain, 2015). From these data we calculated the proportion of agranulocytes out of the total number of leukocytes. Slides containing less than 100 white blood cells were turned into proportions ( $n = 7$  repeats, 5 slides).

### **3.3.2 Statistical analyses**

Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2019), using a log-likelihood ratio test to determine significance of predictors, set at  $p < 0.05$ , in *lme4* 1.1-14 (Bates et al., 2015). The mixed model had a binomial error distribution (link = logit) with the proportion of agranulocytes in the leukocytes as the response variable. We first tested which age transformation (linear or logarithmic) best fitted these data using AICc values, where the relationship between the proportion of agranulocytes and age followed a negative logarithmic pattern ( $\Delta\text{AICc} = 2.9$ ). Logarithmic age was included in the mixed model along with sex, group size, and the interactions between the three. Season was included as a fixed factor and body condition index ( $\log_{10}\text{weight}/\log_{10}\text{body length}$ ; Noonan et al., 2014; Sugianto et al., 2019b) as a fixed covariate since body size and season may affect immune cell concentrations (Downs et al., 2019; Møller et al., 2003; Beaulieu et al., 2017). Body condition index can be interpreted as body-size adjusted body condition (Freckleton, 2002). Cohort,

social group, and slide nested within individual ID were included as random effects. We used parametric bootstrapping ( $n = 5000$ ) to obtain 95% confidence intervals.

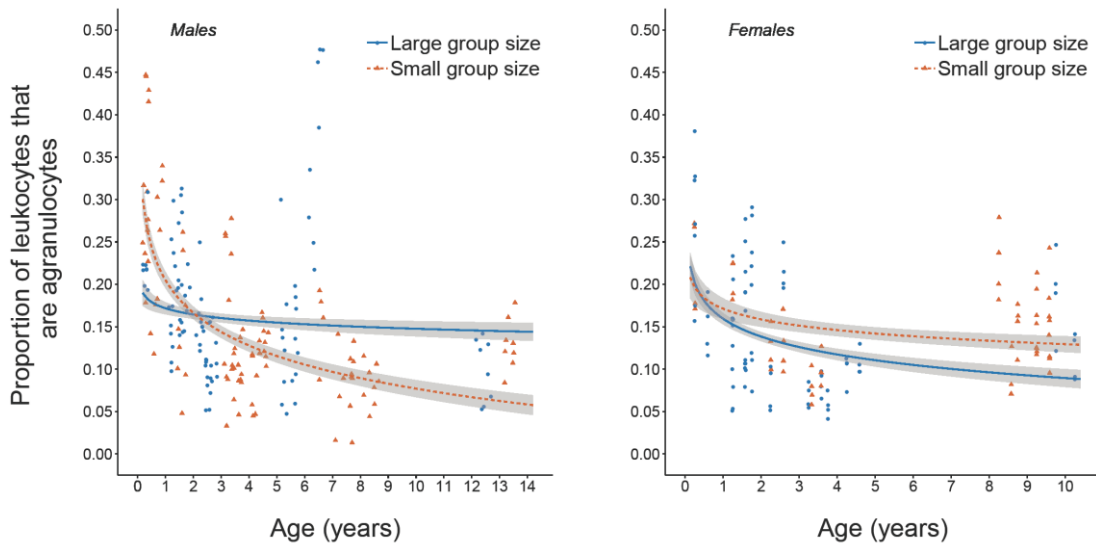
**Table 3.1: Parameter estimates and 95% confidence intervals of fixed effects from a mixed model testing age, sex and group size effects on the proportion of agranulocytes in European badgers.  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval from parametric bootstrapping,  $\chi^2$  = chi-squared value with associated p-value; reference terms in brackets = reference level for factors; \* = interaction. Significant parameters ( $p < 0.05$ ) are in bold.**

Parameter (reference level)	$\beta$	S.E.	95% CI	$\chi^2$	p-value
Intercept	-1.892	0.098	-2.087 to -1.703		
Log age	-0.031	0.097	-0.218 to 0.156	0.143	0.741
Sex (female)	0.099	0.104	-0.111 to 0.313	0.873	0.350
Group size	-0.047	0.086	-0.218 to 0.124	0.300	0.584
Season (Spring)				5.341	0.069
Summer	0.027	0.099	-0.163 to 0.219		
Autumn	0.346	0.154	0.042 to 0.651		
<b>Body condition index</b>	<b>-0.246</b>	<b>0.074</b>	<b>-0.388 to -0.102</b>	<b>9.831</b>	<b>0.002</b>
Log age * Sex (female)	-0.014	0.102	-0.211 to 0.185	0.019	0.889
Log age * Group size	-0.052	0.091	-0.230 to 0.119	0.312	0.556
<b>Sex (female) * Group size</b>	<b>0.255</b>	<b>0.117</b>	<b>0.036 to 0.472</b>	<b>4.176</b>	<b>0.041</b>
<b>Log age * Sex (female) * Group size</b>	<b>0.225</b>	<b>0.104</b>	<b>0.027 to 0.430</b>	<b>4.380</b>	<b>0.036</b>

Random effect estimates (variance): Individual ID ( $1.169 \times 10^{-2}$ ), Slide nested in individual ID ( $1.249 \times 10^{-1}$ ), Social group ( $< 1.000 \times 10^{-12}$ ), Cohort ( $5.026 \times 10^{-3}$ )

### 3.4 Results

There was an interaction between age, group size and sex on the proportion of agranulocytes (Table 3.1). In males, the strength of the logarithmic decrease in the proportion of agranulocytes with age depended on group size: males living in smaller groups had a higher proportion of agranulocytes in early-life which declined with age, whereas there was no clear change with age in males living in larger groups (Figure 3.1). In contrast, in females the proportion of agranulocytes in early-life was similar when living in smaller and larger groups, but with a stronger decrease with age for females living in larger groups (Figure 3.1).



**Figure 3.1: The interplay between age and group size on the proportion of leukocytes that are agranulocytes for males and females. Raw data points are shown. Group size was modelled as a continuous variable in the mixed model, but for visualisation is shown for males in small (range = 1–9;  $n = 99$  repeats; 25 slides; 9 individuals; brown triangles and dashed line) and large (range = 10–16;  $n = 96$  repeats; 24 slides; 8 individuals; blue circles and solid line) groups, and for females in small (range = 1–9;  $n = 52$  repeats; 13 slides; 4 individuals; brown triangles and dashed line) and large (range = 10–16;  $n = 79$  repeats; 20 slides; 6 individuals; blue circles and solid line) groups. X-axis scales differ between plots. Fitted lines represent the model prediction for age interacting with sex and group size, with associated 95% confidence intervals as shaded areas.**

### 3.5 Discussion

We found a relative decrease in the proportion of agranulocytes with age. This may have arisen due to there being quantitatively fewer acquired immunity cells, or because of a greater number of innate cells being produced. In humans, this pattern has been associated with age-related reduction in thymus size (Flores et al., 1999; George and Ritter, 1996), reducing the number of naïve T cells (Fagnoni et al., 2000) and  $CD4^+$  T and  $CD8^+$  subpopulations with age, which has detrimental implications for effective immune responses to new antigens (Goronzy and Weyand, 2005; Effros et al., 2003; Pfister et al., 2006; Kohler et al., 2005; Haynes et al., 2003; Weiskopf et al., 2009). Alternatively, innate immune mechanisms may become more active with age through increased production of pro-inflammatory cytokines (Fagiolo et al., 1993). Such low-grade chronic inflammation in older



individuals has detrimental effects on health and contributes to biological ageing and the development of age-related pathologies (Franceschi et al., 2018). While we cannot provide direct evidence of immunosenescence due to the relative nature of the proportion of agranulocytes, the relative decrease in adaptive immune cells and increase in innate immune cells with age accords with previous studies in the wild (Nussey et al., 2012; Cheynel et al., 2017; Peters et al., 2019). Furthermore, understanding changes in immune cell profiles with age in badgers is important for the interpretation of leukocyte telomere dynamics (van Lieshout et al., 2019). Since granulocytes have longer telomeres than agranulocytes in humans and baboons (Baerlocher et al., 2007; Kimura et al., 2010), any change in telomere length with age in mammals could be due to a change in leukocyte cell composition, or selective loss of leukocytes, with age, and lead to spurious inferences on telomere shortening.

We also provide evidence that social conditions (i.e. group size) have sex-specific effects on changes in individual immune cell profiles with age. In larger groups, early-life exposure to a greater diversity, or higher intensity, of pathogens or greater stress associated with resource or mate competition led to a stronger bias toward innate over adaptive immune cell ratios by age. According to the 'hygiene-hypothesis' (Olszak et al., 2012; von Mutius, 2007; Frölich et al., 2012; Altizer et al., 2003; Côté and Poulin, 1995; Newman et al., 2001), this could subsequently alleviate the detrimental consequences of such pathogens in later-life and thus slow age-related changes in immune cell profiles. In smaller groups, lower exposure to pathogens in early-life can have the opposite effect (Shaw et al., 2010; Goksoo et al., 2011), accelerating changes in immune cell profiles with age. Indeed, we found that the proportion of agranulocytes in early-life was greater in male badgers living in smaller social groups. Moreover, if fewer conspecifics share the pathogen burden, this could lead to a stronger pressure on the immune response and rapid changes in the proportion of agranulocytes.

Even though female badgers exhibited a relative decrease in the proportion of agranulocytes with age, this was not as strong as in males. Possibly, females develop a stronger immune response against pathogens in early-life (i.e. smaller change in the proportion of agranulocytes with age), which would corroborate previous findings in Soay sheep (*Ovis aries*), where males had a steeper decline in agranulocyte proportion with age than did females (Watson et al., 2017). Males, given the polygynandrous mating system of badgers, have high levels of testosterone, particularly compared to other species (Sugianto et al., 2019b), leading to immunosuppression and stronger decreases in adaptive immunity (i.e. agranulocytes) with age (Klein, 2000; Hillgarth and Wingfield, 1997). This accords with sex-specific responses to environmental conditions and associated sex differences in immune responses seen in other species (Klein and Flanagan, 2016; Restif and Amos, 2010).

While males showed stronger relative decreases in the proportion of agranulocytes with age in smaller groups, for females this effect was stronger in larger groups. Since badgers exhibit high levels of extra-group paternity (48%), increasing in proportion to a deficit of within-group candidate fathers, males in smaller groups may be exposed to higher extra-group competition and higher pathogen diversity (Dugdale et al., 2007; Annavi et al., 2014b). In contrast, females compete for resources with other females within their social group (Woodroffe and Macdonald, 1995b), which could lead to detrimental effects of larger group sizes on the proportion of agranulocytes. We were, however, unable to sample individuals until at least three months of age, due to welfare legislation (Protection of Badgers Act, 1992), and thus we cannot rule out the possibility of selective disappearance of individuals with poor innate immune responses. Nonetheless, our results indicate that age-related changes in immune profiles are associated with the social environment and these effects differ between the sexes.

# Supplemental information Chapter 3: Social effects on age-related and sex-specific immune cell profiles in a wild mammal

## Group size estimation:

Group sizes were determined by the number of individuals (cubs and adults) that were present in a social group in a given year. Given high natal philopatry (75.8%), low permanent dispersal rates (19.1%), and high levels of inter-group movements leading to extra-group paternity in badgers (Macdonald et al., 2008), individuals ( $n = 1726$ ) were assigned as a resident of a social group each year, according to the following rules adapted from (Annavi et al., 2014b; Macdonald et al., 2008):

1. Badgers first caught as cub ( $n = 1241$ ) were considered resident in the social group they were first caught, until they subsequently satisfied dispersal rules or were considered dead.
2. Badgers first caught as adults ( $n = 490$ ) were assigned to the lifetime modal social group, until dispersal rules applied. If an individual was captured equally between two groups ( $n = 29$ ), they were assigned to the social group they were initially captured in until dispersal rules applied.
3. Dispersal rules were satisfied when the two most recent captures of an individual (>30 days apart), as well as 1 of 2 captures before, were made in a different social group than the current residential social group. Individuals were resident in the new social group until dispersal rules applied again.

The number of individuals per social group were then calculated as the sum of individuals present in the social group in a given year.

## **Chapter 4: The effects of weather conditions and group size on telomere lengths in a wild mammal**

#### **4.1 Abstract**

Early-life environmental conditions can provide a source of individual variation in senescence patterns, with a potential interplay between the mean of and variability in environmental conditions. Quantification of the consequences of adverse early-life conditions can be achieved using telomere length as a biomarker of senescence. Here, we investigate whether the mean of and variability in early-life weather conditions and group size are associated with early-life telomere length in a wild population of European badgers (*Meles meles*). Cubs experiencing higher mean daily temperatures and higher mean daily rainfall with low variability, had longer early-life telomere lengths (<1 year old), coinciding with food availability and foraging success. We also found an effect of season, where cubs have shorter early-life telomere lengths in winter. Additionally, cubs born in groups with more cubs had longer early-life telomere length, providing no evidence of resource competition, but indicating a social cue for future competition that changes the trade-off between reproductive and somatic investment (which is reflected in early-life telomere length). After sexual maturity, in early adulthood (i.e. 12–36 months) we found no association between telomere length and same-sex adult group size (i.e. intra-sexual competition). We demonstrate that early-life conditions can impact developmental stress, through both the mean of and variability in weather and social conditions, which can have implications for life-history strategies and senescence patterns.

#### **4.2 Introduction**

The early-life environment has lasting effects on individual fitness (Lindstrom, 1999), and may provide a source of variation in individual senescence patterns. Senescence, the decline in performance in older age, has been hypothesised to be faster in individuals living in adverse early-life environments due to different energy allocation trade-offs between early- and later-life in response to the environment (Williams, 1957; Medawar, 1952; Kirkwood and

Rose, 1991). A lower mean quality of the early-life environment, as a particularly sensitive period, might trigger reproductive investment at the expense of somatic maintenance, leading to faster rates of senescence (Kirkwood and Rose, 1991; Lemaitre et al., 2015). Empirical evidence for such detrimental effects of lower mean quality of the early-life environments on senescence, as a consequence of developmental stress, has been found in wild animal populations (Hammers et al., 2013; Reed et al., 2008; Cooper and Kruuk, 2018).

Environmental variability is another form of environmental quality that can shape individual life-history (Nouvellet et al., 2013). It has been hypothesised that individuals in temporally stochastic environments should adopt a bet-hedging strategy, with higher somatic than reproductive investment, resulting in longer lifespans in variable conditions (Morris et al., 2008; Wilbur and Rudolf, 2006; but see Shpak, 2007). However, environmental fluctuations may have age-specific effects on different genotypes, and faster rates of senescence have been predicted in variable environmental conditions (Cotto and Ronce, 2014). Since variability in weather conditions is predicted to increase in the future (IPCC, 2014), it is important to understand the implications of variable early-life conditions on life-histories. The mean early-life conditions experienced may interact with the variability around the mean where, for example, high variability around a low mean temperature accelerates malaria transmission in mosquitos (Paaijmans et al., 2010), or where the interaction between the mean of and variability in temperature impacts population growth and survival in *Drosophila melanogaster* (Bozinovic et al., 2011). The interplay between the mean of and variability in early-life environmental conditions, such as the abundance and variation in food, foraging success and thermal stress for young individuals (Webb and King, 1984; Noonan et al., 2015; Nouvellet et al., 2013), can potentially impact developmental stress, longevity and senescence.

Social conditions in early-life can also shape life-history due to increased competition for food. For example, female red deer (*Cervus elaphus*) that experienced high levels of

resource competition in early-life showed faster rates of reproductive senescence (Nussey et al., 2007a). Such competition for food may be even stronger in group living species due to a greater number of offspring competing for food and foraging space. Additionally, developing individuals can use the number of contemporaries as a cue for future competition and anticipate development accordingly through developmental plasticity (Bretman et al., 2016; Brown and Brown, 2003; Lemaitre et al., 2011; Kasumovic and Brooks, 2011). However, more parents and parental care can alleviate detrimental effects of competition for food in offspring, and enhance offspring development with slower senescence rates (Ridley, 2007; Russell et al., 2007).

The effects of social conditions on senescence may also become apparent after sexual maturity, when individuals compete for mating opportunities (Beirne et al., 2015; Andersson, 1994). In polygynous species, sex differences in senescence may be attributable to intense intra-sexual competition between males (Williams, 1957; Promislow, 1992; Clutton-Brock and Isvaran, 2007). Males invest in intra-sexual competition and reduce investment in self-maintenance (Kirkwood and Rose, 1991). This may select for shorter lifespan and faster senescence in males, compared to females (Williams, 1957; Clutton-Brock and Isvaran, 2007). While this prediction has been challenged (Promislow, 2003; Graves, 2007; Bonduriansky et al., 2008), and sex-specific senescence may be trait-dependent with respect to the underlying physiological processes (Nussey et al., 2009), increases in male-biased actuarial senescence in polygynous and sexual dimorphic species exist (Promislow, 1992; Clutton-Brock and Isvaran, 2007). While social effects may also contribute to senescence in females (Woodroffe and Macdonald, 1995b; Sharp and Clutton-Brock, 2011), such sex-specific social effects on senescence are expected to be greater in males (Clutton-Brock and Isvaran, 2007; Maklakov and Lummaa, 2013; Bonduriansky et al., 2008).

One way to quantify the effects of early-life environmental conditions on senescence is through telomere length, which functions as a biomarker of senescence (Monaghan and

Hausmann, 2006; Monaghan, 2010). Telomeres are highly conserved nucleoprotein structures at the end of chromosomes consisting of a non-coding sequence (5'-TTAGGG-3') and shelterin proteins (Blackburn, 2000). Telomeres maintain genomic integrity by preventing chromosome degradation and fusion of ends by forming T-loops (de Lange, 2004). Generally, telomeres shorten with each cell replication due to the end-replication problem (Olovnikov, 1973), but telomere shortening can be accelerated through stressors (Heidinger et al., 2012; Epel et al., 2004) and potentially oxidative damage (Reichert and Stier, 2017; von Zglinicki, 2002; but see Boonekamp, 2017). Telomeres can, however, also elongate due to the enzyme telomerase (Blackburn et al., 1989) and other telomere-elongation pathways (Cesare and Reddel, 2010; Mendez-Bermudez et al., 2012). Cells with critically short telomeres then enter replicative senescence, where the accumulation of senescent cells can impair tissue function due to reduced renewal capacity (Campisi, 2005; Campisi and di Fagagna, 2007).

Extensive evidence links adverse early-life conditions to shorter telomeres (McLennan et al., 2016; Watson et al., 2015; Mizutani et al., 2013), which are associated with reduced survival probability (Wilbourn et al., 2018). Additionally, there is evidence for conspecific resource competition in early-life leading to greater telomere shortening (Boonekamp et al., 2014; Nettle et al., 2015; Stier et al., 2015), and shorter telomere lengths (Cram et al., 2017). However, little is known about the effects of the mean of and variability in early-life weather conditions on early-life telomere length. Additionally, whether increased intra-sexual competition (e.g. higher local densities of same-sex individuals) is associated with shorter telomere lengths remains to be tested.

To test the effects of early-life environmental conditions on telomere length, we use a long-term dataset from a wild population of European badgers (*Meles meles*; henceforth 'badgers'). Badgers show reproductive senescence with males having a later onset, but faster rate of senescence than females (Dugdale et al., 2011b). Additionally, early-life telomere



length is a biomarker of senescence in badgers (van Lieshout et al., 2019). Badgers are natally philopatric and form large social groups (mean group size = 11.3, range = 2–29; da Silva et al., 1994) with latrine-marked borders (Buesching et al., 2016; Delahay et al., 2000), although they do transgress these borders when foraging (Ellwood et al., 2017; Noonan et al., 2015) without any sex difference in foraging niche (Robertson et al., 2014). In terms of food availability, badgers mainly feed on earthworms (*Lumbricus terrestris*; Kruuk and Parish, 1981; Johnson et al., 2001). Earthworms are sensitive to microclimatic conditions (Gerard, 1967; Edwards and Bohlen, 1996; Newman et al., 2017), making their abundance and distribution highly dependent on weather conditions and resulting in high-population densities in areas with damp conditions where earthworms are available (Kruuk, 1978b; Macdonald et al., 2015; Newman et al., 2017). Badgers preferentially forage on earthworms, but do change to alternative food sources (e.g. berries) when weather patterns make earthworms scarcely available (Macdonald et al., 2010). Foraging success in adverse weather conditions is lower, either due to thermal stress when foraging in cold and wet conditions or due to remaining in thermally-stable setts (Nouvellet et al., 2013; Noonan et al., 2014; Noonan et al., 2018). Higher mean daily temperature and mean daily rainfall have been positively associated with both juvenile and adult survival probability as well as recruitment, whereas the variability in these metrics has detrimental consequences for survival and recruitment (Nouvellet et al., 2013; Macdonald et al., 2010).

Badger cub growth rates and maturation depend on natal social conditions, with early developers reaching sexual maturity at 11 months old and late developers at 22–26 months old (Sugianto et al., 2019b). However, the effects of the numbers of cubs in the natal group appear to be stronger for male cubs (Sugianto et al., 2019a; Sugianto et al., 2019b). Badgers are polygynandrous and have high levels of extra-group paternity (48%), with 86% of extra-group offspring sired by neighbouring group males (Dugdale et al., 2007; Annavi et al., 2014b). There is reproductive skew, where 27% ( $\pm 6\%$  SE) and 31% ( $\pm 6\%$  SE) of adult males

and females, respectively, breed per year (Dugdale et al., 2007; Dugdale et al., 2008). Badgers exhibit male-biased mortality (Bright Ross; unpublished data) and a slight male-biased sexual dimorphism that can be ascribed to differential patterns of development among males and females (Noonan et al., 2016; Sugianto et al., 2019a).

Here, we investigate the relationships between early-life conditions and relative leukocyte telomere length (RLTL), by testing whether: (i) adverse weather conditions, as a proxy for food availability and the thermal stresses cubs are exposed to, are associated with shorter early-life RLTL; (ii) adverse early-life social conditions, i.e. cubs born in groups with more cubs, as a proxy for food competition, are associated with shorter early-life RLTL, and whether such effects are stronger for males; and (iii) adverse social conditions after sexual maturity, i.e. larger same-sex adult group size for females and, for males, more within-group and neighbouring-group adult (>1 year old) males, are associated with shorter RLTL in early adulthood.

### **4.3 Methods**

#### **4.3.1 Study population and trapping**

We conducted this study in a high-density population of European badgers (mean $\pm$ SE = 36.4 $\pm$ 2.55 badgers/km<sup>2</sup>; Macdonald et al., 2009) in Wytham Woods, Oxfordshire, UK (51°46'24"N, 1°20'04"W); a 424 ha mixed semi-natural woodland surrounded by mixed arable and permanent pasture (Macdonald and Newman, 2002). Wytham Woods had a mean annual temperature of 10.6 °C ( $\pm$ 5.5 SD) and mean annual precipitation of 684 ( $\pm$ 129 SD) mm between 1987–2010. The population consisted of 19 $\pm$ 2 (mean  $\pm$  95% CI; range = 14–26; Dugdale et al., 2008) mixed-sex social groups (Johnson et al., 2002; Newman et al., 2011) during the period that we analysed, 1987–2010. The Wytham badger population is geographically discrete (Macdonald et al., 2009) with only ca. 3% annual immigration/emigration per year (Macdonald and Newman, 2002).

The badger population has been trapped systematically since 1987 over two weeks in May–June (Spring), August–September (Summer) and November (Autumn), with trapping in January (Winter) in focal years. Badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren et al., 2005). Upon first capture, badgers were tattooed with a unique number on the left inguinal region for permanent identification. Sex, age class (cub <1 year old; adult  $\geq$ 1 year old), capture date and social group were recorded. Age of badgers was defined by the number of days since the 14<sup>th</sup> of February, reflecting the February birth peak (Yamaguchi et al., 2006). Age of badgers first caught as adults was inferred from tooth wear (da Silva and Macdonald, 1989; Macdonald et al., 2009); badgers with a tooth wear of 2 were assumed to be 1-year old adults (van Lieshout et al., 2019). Whole blood samples were collected from anaesthetised badgers through jugular venipuncture into vacutainers with an EDTA anticoagulant, and stored immediately at -20°C. Badgers were released after full recovery from anaesthesia. Additionally, bait-marking (Macdonald and Newman, 2002; Delahay et al., 2000) was conducted periodically to delimit group range sizes and configure social groups.

#### **4.3.2 Telomere analyses**

Genomic DNA was extracted from whole blood samples ( $n = 841$  samples; 562 badgers) using the DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol, with adjustments using 125  $\mu$ l of anticoagulated blood and a double elution step (2x 75  $\mu$ l AE buffer). We checked DNA integrity by running a random selection of DNA extracts (ca. 20%) on agarose gels to ensure high molecular weight. DNA concentration of all samples was quantified using the Fluostar Optima fluorometer (BMG Labtech, Ortenberg, Germany) and standardized to 20 ng/ $\mu$ l, after which samples were stored at -20 °C. We used monochrome multiplex quantitative PCR (MMqPCR; Cawthon, 2009) analysis to measure relative leukocyte telomere lengths. This measure represents the mean telomere length

across cells in a sample, which is calculated as the abundance of telomeric sequences relative to a reference gene where both are analysed in the same well. A detailed description of the MMqPCR analysis is given in van Lieshout et al. (2019).

#### **4.3.3 Weather conditions**

Weather metrics, as a proxy for developmental stress in terms of earthworm food availability and thermoregulatory costs while foraging (Nouvellet et al., 2013; Noonan et al., 2014; Macdonald et al., 2010), were calculated using weather data (mean daily temperature and total daily rainfall) from the Radcliffe Meteorological Station, School of Geography, University of Oxford. No critical window for weather predictors in relation to early-life RLTL could be differentiated from the candidate windows using the sliding window approach (van de Pol et al., 2016; Table S4.1), and we therefore used a biologically sensible window that reflects the strongest developmental stress on individuals (February–May). This period was selected as cubs are born in February (Yamaguchi et al., 2006) and exhibit high growth rates depending on food availability and social conditions (Sugianto et al., 2019a).

Weather metrics were calculated from 1987 to 2010, where two metrics for temperature were calculated as the: i) mean of mean daily temperature over February to May; and, ii) variability in daily temperature over February to May – since daily temperatures within a year follows a sinusoidal pattern, variability in temperature was calculated as the sum of squared residuals in deviation around the daily predicted temperatures (i.e. distance from sinusoidal curve to the daily temperature). Rainfall did not follow clear seasonal trends and was characterised simply as the: i) mean daily rainfall over February to May, and ii) coefficient of variation in daily rainfall over February to May (see Nouvellet et al., 2013 for a detailed description).

#### 4.3.4 Group sizes

Group sizes were determined by the number of individuals (cubs and adults) that were present in a social group in a given year. Given high natal philopatry (35.8%), low permanent dispersal rates (19.1%), and high levels of inter-group movements (Macdonald et al., 2008), individuals ( $n = 1726$ ) were assigned as a resident of a social group each year, according to the following rules adapted from Annavi et al. (2014b) and Macdonald et al. (2008):

1. Badgers first caught as cubs ( $n = 1241$ ) were considered resident in the social group they were first caught in, until they subsequently satisfied dispersal rules or were considered dead.
2. Badgers first caught as adults ( $n = 490$ ) were assigned to their lifetime modal social group, until dispersal rules applied. If an individual was captured equally between two groups ( $n = 29$ ), they were assigned to the social group they were initially captured in until dispersal rules applied.
3. Dispersal rules were satisfied when the two most recent captures of an individual (>30 days apart), as well as 1 of 2 captures before, were made in a different social group than the current resident group. Individuals were then resident in the new social group until dispersal rules applied again.

The number of individuals per social group were then calculated as the sum of individuals present in the social group in a given year. These measures were separated by age class (i.e. cub/adult) and sex (i.e. male/female) to determine sex- and age-specific group sizes.

To measure intra-sexual competition in females, we calculated female adult group sizes, as females compete with other within-group females (Woodroffe and Macdonald, 1995b). However, for males, extra-group paternity is high (48%) and affected by the number of within-group and extra-group candidate fathers (Annavi et al., 2014b), so we combined both the number of within-group males and neighbouring-group males. The mean number of cubs in a social group was 4.2 ( $\pm 2.2$  SD; range 1–10), the mean number of female adults in

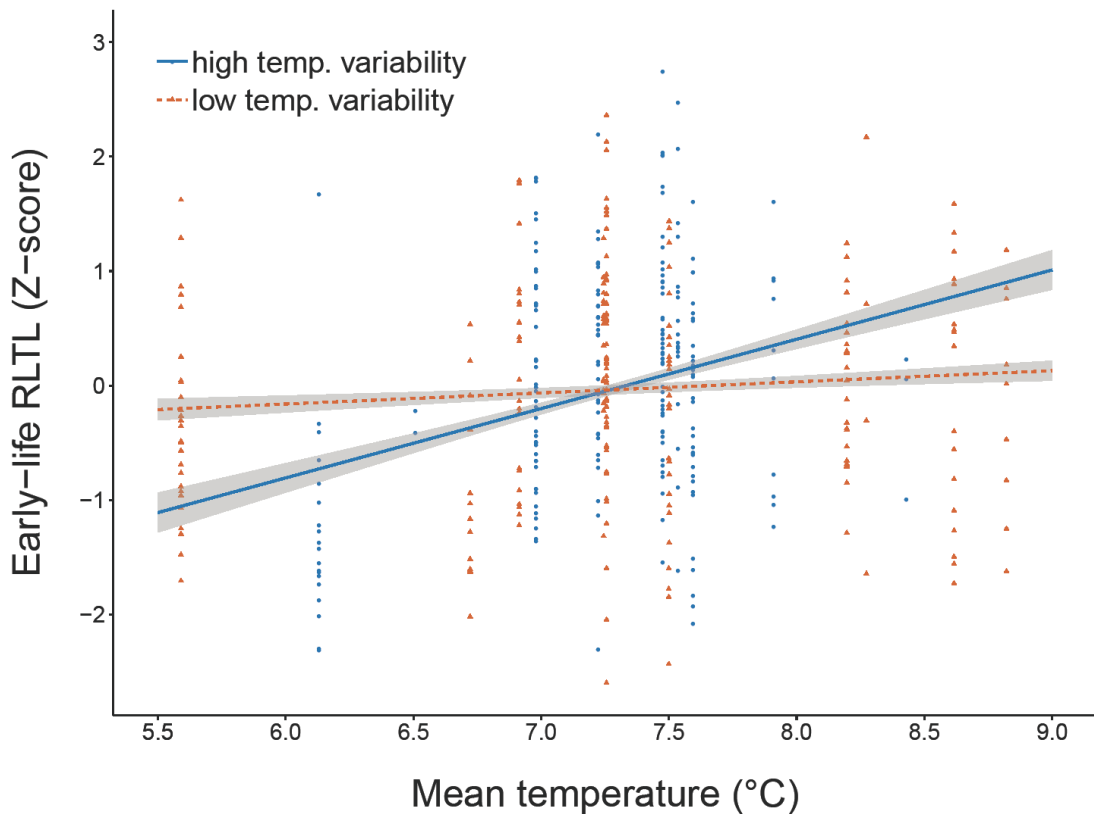
a social group was 7.1 ( $\pm 3.4$  SD; range 1–17) and the mean number of male adults in focal plus neighbouring social groups was 30.0 ( $\pm 11.1$  SD; range 3–59).

#### 4.3.5 Statistical analyses

Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2019) with RLTL as the response variable, which was first square-root transformed and then turned into Z-scores (Verhulst, 2019). All General Linear Mixed Models (GLMMs) included natal social group, qPCR-plate and row on qPCR-plate as random effects. Significance of fixed effects was determined with log-likelihood ratio tests, set at  $P < 0.05$ . Model fit was assessed using standard residual plot techniques and fixed effects were checked for collinearity ( $VIF < 3$ ).

We first tested whether conditions experienced as a cub ( $< 1$  year old) affected early-life RLTL. In a GLMM with early-life RLTL as the response variable and age (months), cohort and season as fixed effects, we determined that early-life RLTL did not vary with age ( $n = 435$ ,  $\chi^2 = 0.060$ , d.f. = 1,  $P = 0.806$ ), and excluded age from the subsequent analysis. The effects of first-year conditions on early-life RLTL were then modelled with early-life RLTL as the response variable ( $n = 435$ , samples; 435 badgers) and the number of cubs in the natal group, sex and its interaction as fixed effects due to sex-specific developmental pathways (Sugianto et al., 2019a). We also included the number of adult females in the social group as a covariate to account for the number of mothers that may provide care for offspring, and season as a fixed factor. In the same model, to test effects of early-life conditions, we included an interaction between mean daily temperature and variability in daily temperature, and an interaction between mean daily rainfall and the coefficient of variation in daily rainfall as fixed effects, and cohort as an additional random effect. To confirm whether the number of contemporary cubs provide a social cue we then removed the interaction between sex and the number of cubs in the natal group.

Badgers generally reach sexual maturity at 1-year old (Dugdale et al., 2007), but may not become reproductively active until 2 years of age (Sugianto et al., 2019b) and therefore first produce offspring when they are 3 years of age, due to delayed implantation. We therefore examined whether same-sex adult group sizes were reflected in RLTL in early adulthood (i.e. 12–36 months old). In a GLMM with RLTL in early adulthood as the response variable with age thresholds (see van Lieshout et al., 2019), cohort and season as fixed effects and year and individual ID as additional random effects, we determined that RLTL did not vary with age ( $n = 406$ , age threshold 1,  $\chi^2 = 2.561$ , d.f. = 1,  $P = 0.110$ ; age threshold 2,  $\chi^2 = 3.321$ , d.f. = 1,  $P = 0.068$ ), and excluded age from the subsequent analysis. The effects of same-sex adult group size on RLTL in early adulthood were modelled with RLTL in early adulthood as the response variable ( $n = 406$  samples; 329 individuals). Same-sex adult group size (within-group for females and within- plus neighbouring-group for males), sex and its interaction (to model differential strength in intra-sexual competition among the sexes) age at last capture (to control for selective disappearance), and season were included as fixed effects, and cohort, year and individual ID as additional random effects.



**Figure 4.1: The interaction between mean daily temperature and variability in daily temperature from February to May on early-life relative leukocyte telomere length (RLTL). Raw data points ( $n = 435$ ) are shown; these were modelled as continuous fixed effects but for visualisation are shown for cubs experiencing low ( $n = 223$ ; brown, triangle, dashed) or high temperature variability ( $n = 212$ ; blue, circle, solid). Fitted lines represent the model prediction for mean daily temperature interacting with variability in daily temperature, with associated 95% confidence intervals as shaded areas.**

#### **4.4 Results**

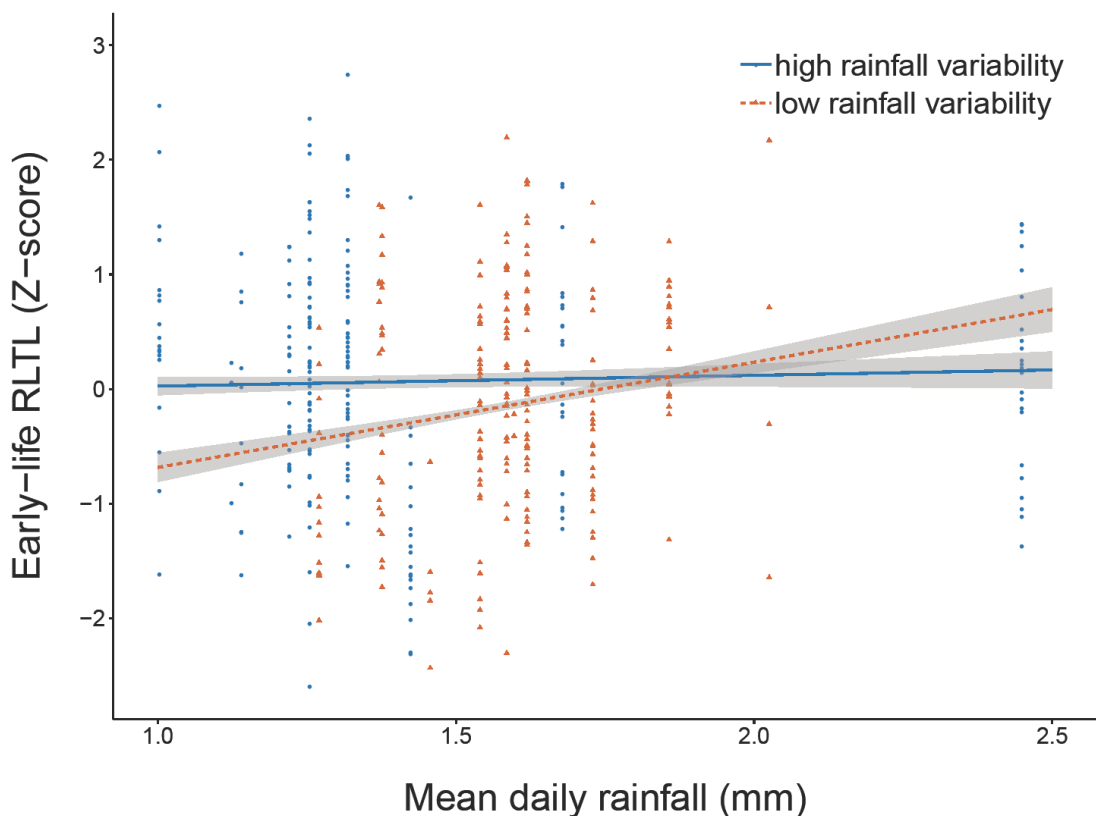
##### **(a) Effects of weather and group size on early-life RLTL**

We found a positive interaction between mean daily temperature and variability in daily temperature on early-life RLTL (Figure 4.1; Table S4.2). Cubs experiencing colder conditions had shorter early-life RLTL compared to warmer conditions (Figure 4.1; Table S4.2). Yet, cubs experiencing higher mean daily temperatures benefitted from high variation in daily temperature, whereas with lower mean daily temperatures, variation in daily temperature had a detrimental effect on early-life RLTL (Figure 4.1; Table S4.2). There was also an effect



of season, with cubs having shorter early-life telomere length in winter compared to spring (Table S4.2; Figure S4.1).

We also found a negative interaction between mean daily rainfall and the coefficient of variation in daily rainfall on early-life RLTL (Figure 4.2; Table S4.2). Cubs experiencing lower mean daily rainfall benefitted from high variation in daily rainfall, whereas with higher mean daily rainfall, variation in daily rainfall had a detrimental effect on early-life RLTL (Figure 4.2; Table S4.2).

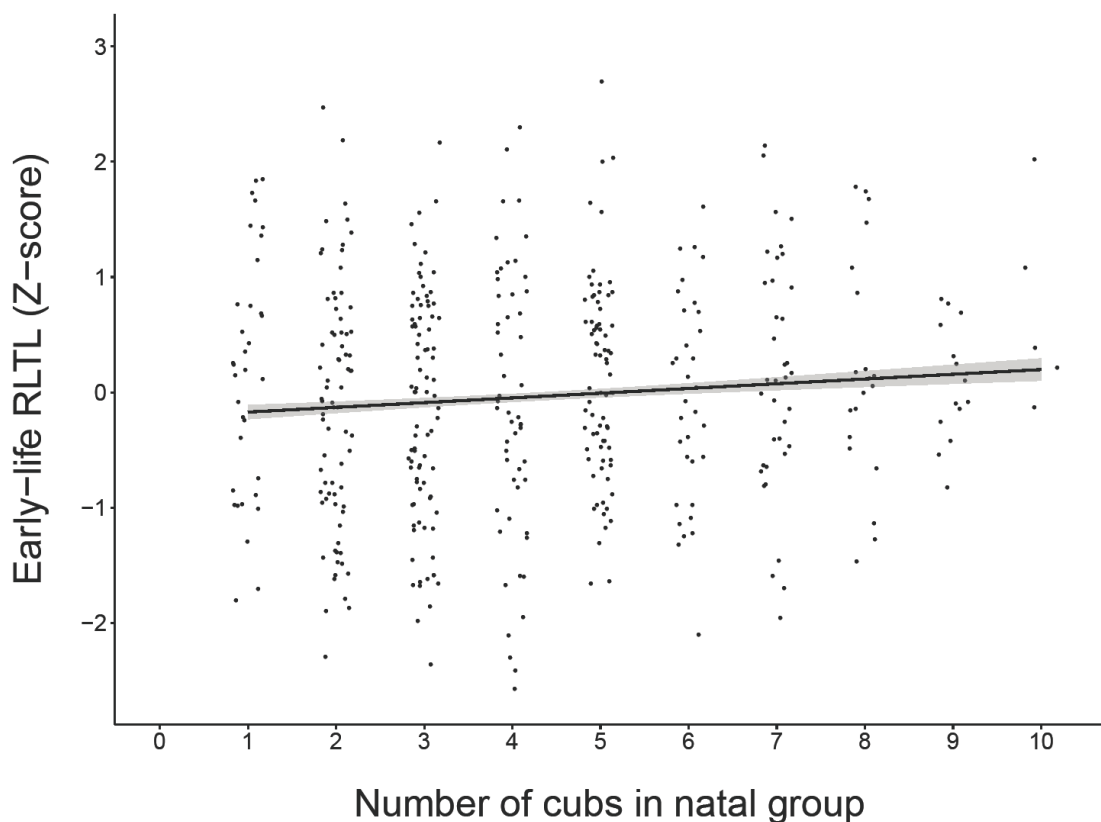


**Figure 4.2:** The interaction between mean daily rainfall and the coefficient of variation in daily rainfall from February to May on early-life relative leukocyte telomere length (RLTL). Raw data points ( $n = 435$ ) are shown and modelled as a continuous variable in the mixed model, but for visualisation are shown for cubs shown experiencing low ( $n = 211$ ; brown, triangle, dashed) or high variation in daily rainfall ( $n = 224$ ; blue, circle, solid). Fitted lines represent the model prediction for mean daily rainfall interacting with coefficient of variation in daily rainfall, with associated 95% confidence intervals as shaded areas.

We found no evidence for a sex-specific effect of the number of cubs in the natal group on early-life RLTL (Table S4.2). However, we found a positive relationship between the number of cubs in the natal group and early-life RLTL, while accounting for the number of adult females in the group and early-life weather conditions, indicating that the number of cubs provides a social cue of future competition (Figure 4.3; Table S4.3).

#### **(b) Effects of same-sex group size on RLTL in early adulthood**

We found no evidence of same-sex adult group size effects on RLTL in early adulthood for females or males (Table S4.4).



**Figure 4.3: The relationship between the number of cubs in the natal group and early-life relative leukocyte telomere length (RLTL). Raw data ( $n = 435$ ) are shown as open circles with number of cubs in natal group as integers, but jittered for clarity on the amount of data. The fitted line represents the regression from the mixed model, and the 95% confidence intervals as the shaded area.**

## 4.5 Discussion

Our results show that badger cubs born in warm and wet conditions, with little variation in rainfall, had longer early-life RLTL. We also found no evidence for sex-specific effects of the number of cubs in the natal group on early-life RLTL, but we found that cubs born in groups with more cubs had longer early-life RLTL. Additionally, we found no evidence of intra-sexual competition effects (in terms of the number of within-group adult females, or both within-group and extra-group adult males) on RLTL in early adulthood.

Cubs that experienced higher mean daily temperatures, independent of daily temperature variability or with high temperature variability, had longer early-life RLTL, whereas the benefits of mean daily rainfall were dependent on the variation in daily rainfall for early-life RLTL. Since badgers feed mainly on earthworms, which are available in warm and wet conditions, high mean daily temperature and rainfall result in greater food availability (Kruuk, 1978b; Newman et al., 2017). Even though weather conditions experienced as cubs can affect early-life RLTL through thermoregulatory costs while foraging (Webb and King, 1984; Nouvellet et al., 2013), accelerometry tracking has shown that, particularly fatter, badgers will remain in the thermally-stable setts during adverse weather conditions and avoid a net-negative energy return of foraging (Noonan et al., 2015; Noonan et al., 2018; Tsunoda et al., 2018) by going into torpor (Noonan et al., 2014). Individuals born in relatively warm and wet conditions may therefore achieve a positive energy balance, which can be invested in either growth or somatic maintenance (Kirkwood, 1977; Kirkwood and Rose, 1991), and result in slower patterns of senescence (e.g. Hammers et al., 2013). Additionally, we found seasonal effects, with shorter early-life telomere length in the winter season because of sub-optimal foraging conditions and food availability (Macdonald and Newman, 2002; Newman et al., 2017), and when badgers go into torpor (Noonan et al., 2014). Thus, favourable weather conditions, i.e. higher mean daily temperature and rainfall,

associated with longer early-life RLTL indicate reduced developmental stress, greater longevity and slower senescence patterns.

The effect of the mean of early-life environmental conditions on early-life RLTL (i.e. mean daily temperature and mean daily rainfall) depended on the variability in early-life environmental conditions. In environments with a higher mean daily temperature or daily rainfall, greater weather variability can reduce the predictability of food availability and impact foraging activity (Noonan et al., 2018), and result in maladaptation which has been associated with faster rates of senescence (Cotto and Ronce, 2014). This is in line with previous findings where badgers showed higher cub recruitment and survival probability in conditions with restricted deviation from the mean yearly temperature (Nouvellet et al., 2013). However, in conditions with lower mean daily rainfall, variability may benefit the individual by providing a temporal increase in resource availability and foraging conditions. Individuals can then modulate their energy trade-offs in response to the mean of and variability in environmental conditions (Erikstad et al., 1998; Weimerskirch et al., 2001; Reid et al., 2003) and adopt a bet-hedging strategy until environmental conditions are favourable (Morris et al., 2008; Wilbur and Rudolf, 2006). The interplay between variability and mean environmental conditions changes the quality of the environment which shapes individual fitness, and since variability is likely to increase under current climate change (IPCC, 2014), this can impact ecological and individual resilience. Understanding the consequences of variability, depending on the mean environmental conditions, on RLTL and life-history is not only important in terms of climate change and habitat fragmentation, but may also provide a source of variation in senescence patterns.

Besides weather conditions related to food availability, we show no sex-specific effects of the number of cubs on early-life telomere length. There is therefore no clear evidence that males are more susceptible to social stress than females. However, we found that cubs born in groups with more cubs had longer early-life RLTL, after controlling for the

potential for greater care in groups with more mothers (Dugdale et al., 2010). This is in contrast with the current consensus around early-life social conditions that competition for food has detrimental consequences for telomere length and telomere shortening (Cram et al., 2017; Boonekamp et al., 2014; Nettle et al., 2015). While badger cubs may have access to sufficient food that does not lead to developmental constraint, these studies were also able to measure telomere length within the first month of life. In contrast, we were unable to sample individuals until at least 3 months of age, due to welfare legislation (Protection of Badgers Act, 1992), when the weakest cubs could have already succumbed and therefore result in different group sizes; thus, we measured RLTL from three to twelve months of age. With little knowledge of pre-emergence group dynamics in badgers, underground resource competition before capture may lead to similar patterns of early-life RLTL as found in our results. However, cubs may also use the number of contemporaries as a cue of future competition (Bretman et al., 2016; Kasumovic and Brooks, 2011). Such socially cued anticipatory plasticity may be reflected in early-life telomere length, where a smaller number of cubs can provide a cue to accelerate development to sexual maturity to utilise the opportunity to reproduce, as a form of developmental plasticity (Bateson et al., 2004; Stevenson and Bancroft, 1995; Bretman et al., 2016). This is in line with badger cubs, particularly males, born in groups with fewer cubs having faster development, growth rates and reaching sexual maturity earlier (Sugianto et al., 2019b). Such reproductive investment at the expense of somatic maintenance, reflected in early-life RLTL, indicates that social cues in early-life have consequences for senescence through developmental plasticity.

Social conditions in early adulthood, however, indicated no association between same-sex adult group size and RLTL in early adulthood. While in polygynous species a stronger competition among males for mating opportunities can be expected (Trivers, 1972; Clutton-Brock and Isvaran, 2007), Wytham badgers only exhibit a slight sexual dimorphism (Johnson and Macdonald, 2001) and male-biased mortality (Bright Ross; unpublished data).

Intra-sexual competition may therefore not be strong in badgers, where the high levels of polygynandry, promiscuity and repeated mounting behaviour may have evolved to reduce male-male aggression and infanticide from males (Dugdale et al., 2011a; Wolff and Macdonald, 2004). The post-copulatory mechanisms in badgers regarding cryptic female choice (i.e. delayed implantation, superfecundation and superfetation) may promote sperm competition, mask paternity and increase genetic diversity and compatibility rather than direct intra-sexual competition (Birkhead and Pizzari, 2002). Such genetic diversity is enhanced in badgers by high levels of extra-group paternity, where particularly the number of within-group and extra-group fathers (neighbouring groups) define the relative proportion of extra-group offspring (Annavi et al., 2014b; Dugdale et al., 2007). Additionally, although there is reproductive skew in males, where males with a higher body condition index attain reproductive success, this is not strong and achieving reproductive success may thus depend on other biological or environmental factors (Dugdale et al., 2008). Even though there is substantial variation in reproductive senescence between male and female badgers (Dugdale et al., 2011b), we found no evidence that variation in senescence patterns, reflected in early-life telomere length, is due to intra-sexual competition in early adulthood. Our study contrasts with a study in a badger population studied at Woodchester Park (Beirne et al., 2015), where males show higher rates of body mass senescence than females due to downstream effect of intra-sexual competition in early adulthood. While there is no evidence of direct effects of intra-sexual competition in early adulthood on telomere length, there may be downstream effects on body mass senescence.

In conclusion, our findings support the view that the mean weather conditions experienced, and the interaction with variability in weather conditions, can impact on RLTL and senescence. Further studies investigating individual variation in senescence should therefore not only focus on the mean conditions but also the variability therein to understand the selective pressures on senescence. Additionally, social conditions in early-

life can constrain development when food is limited (Boonekamp et al., 2014; Nettle et al., 2015), but we provide evidence that social cues in early-life can lead to plastic responses in anticipation of future competition. Such developmental plasticity is important in shaping life-history trade-offs and as a consequence can impact the rates of senescence. However, we found no evidence for effects of intra-sexual competition on RLTL in early adulthood. The mean early-life conditions, the variability therein, and the potential for plastic responses in anticipation of such conditions under current climate change has strong implications for developmental stress and senescence patterns.

## Supplemental information Chapter 4: The effects of weather conditions and group size on telomere lengths in a wild mammal

### Supplementary tables

Table S4.1: Estimates for the optimal climatic window from the baseline model (without climatic predictors), with early-life relative leukocyte telomere length (RLTL; <1 year old) as the response variable, the number of cubs in the natal social group, female adult group size and season as fixed effects, and cohort, natal social group, qPCR plate and row on qPCR plate as random effects. Windows were estimated for mean ( $\mu$ ) daily temperature, variability ( $\sigma^2$ ) around daily predicted temperature, total ( $\Sigma$ ) daily rainfall and coefficient of variation (CV) in total daily rainfall. Absolute windows were tested, with a range of 333–0 days before the 31<sup>st</sup> of December in the corresponding year of measurement, with daily intervals. The response function was linear ( $n = 435$  samples, 435 badgers over 20 years). The top models for each variable are shown, with ‘Open’ indicating the start of the climatic window, and ‘Close’ as the end of the window.  $\Delta\text{AICc}$  compared to the null model (a lower value represents a better model fit), with  $\hat{\theta}$  = model estimate and S.E = standard error. Randomisations ( $n = 100$ ) were conducted to determine the likelihood ( $p$ -value) of candidate signals occurring by chance, where  $p > 0.05$  indicates that the model could not differentiate an optimal window out of all candidate windows.

Model	Variable	$\Delta\text{AICc}$	Open	Close	$\hat{\theta}$	S.E.	$p$ -value
1	$\mu$ temp.	-7.20	214	175	0.05938	0.00306	0.268
2	$\sigma^2$ temp.	-2.46	252	40	0.35969	0.00315	0.903
3	$\Sigma$ rainfall	-10.87	183	179	-0.02596	0.00308	0.866
4	CV rainfall	-4.93	183	179	-0.00023	0.00306	0.461



**Table S4.2: Parameter estimates and 95% confidence intervals of fixed effects from a mixed model testing group size and weather effects on early-life relative leukocyte telomere length (Z-score) in European badgers.  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval,  $\chi^2$  = chi-squared value; reference terms in brackets = reference level for factors; \* = interaction. Significant parameters ( $p < 0.05$ ) are in bold.**

<b>Parameter (reference level)</b>	<b><math>\beta</math></b>	<b>S.E.</b>	<b>95% CI</b>	<b>X<sup>2</sup></b>	<b>p-value</b>
Intercept	-0.050	0.113	-0.219 to 0.169		
Number of cubs in natal group	0.093	0.070	-0.045 to 0.225	1.842	0.171
Sex (Female)	-0.026	0.089	-0.188 to 0.159	0.027	0.871
Number of adult females in natal group	-0.042	0.048	-0.134 to 0.060	0.638	0.424
<b>Season (Spring)</b>				<b>12.61</b>	<b>0.006</b>
<b>Summer</b>	<b>0.095</b>	<b>0.105</b>	<b>-0.125 to 0.287</b>		
<b>Autumn</b>	<b>-0.038</b>	<b>0.189</b>	<b>-0.458 to 0.279</b>		
<b>Winter</b>	<b>-0.827</b>	<b>0.269</b>	<b>-1.416 to -0.375</b>		
<b>Mean temperature</b>	<b>0.250</b>	<b>0.078</b>	<b>0.138 to 0.377</b>	<b>11.79</b>	<b>&lt;0.001</b>
<b>Daily temperature variability</b>	<b>0.129</b>	<b>0.080</b>	<b>0.009 to 0.255</b>	<b>4.320</b>	<b>0.038</b>
<b>Mean daily rainfall</b>	<b>0.256</b>	<b>0.083</b>	<b>0.121 to 0.385</b>	<b>11.72</b>	<b>&lt;0.001</b>
<b>Coefficient of variation in daily rainfall</b>	<b>0.181</b>	<b>0.082</b>	<b>0.061 to 0.315</b>	<b>7.106</b>	<b>0.008</b>
Number of cubs in natal group *	0.028	0.089	-0.142 to 0.204	0.122	0.726
Sex (Female)					
<b>Mean temperature * Daily temperature variability</b>	<b>0.258</b>	<b>0.065</b>	<b>0.168 to 0.381</b>	<b>14.97</b>	<b>&lt;0.001</b>
<b>Mean daily rainfall *</b>	<b>-0.355</b>	<b>0.113</b>	<b>-0.555 to -0.187</b>	<b>11.89</b>	<b>&lt;0.001</b>
<b>Coefficient of variation in daily rainfall</b>					

Random effect estimates (variance): Plate ( $4.622 \times 10^{-2}$ ), Row ( $6.316 \times 10^{-3}$ ), Natal social group ( $1.010 \times 10^{-2}$ ), Cohort ( $5.694 \times 10^{-2}$ ), Residual ( $7.733 \times 10^{-1}$ )

**Table S4.3: Parameter estimates and 95% confidence intervals of fixed effects from a mixed model testing group size and weather effects on early-life relative leukocyte telomere length (Z-score) in European badgers, but without the interaction between the number of cubs in the natal group and sex.  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval,  $\chi^2$  = chi-squared value with associated p-value; reference terms in brackets = reference level for factors; \* = interaction. Significant parameters ( $p < 0.05$ ) are in bold.**

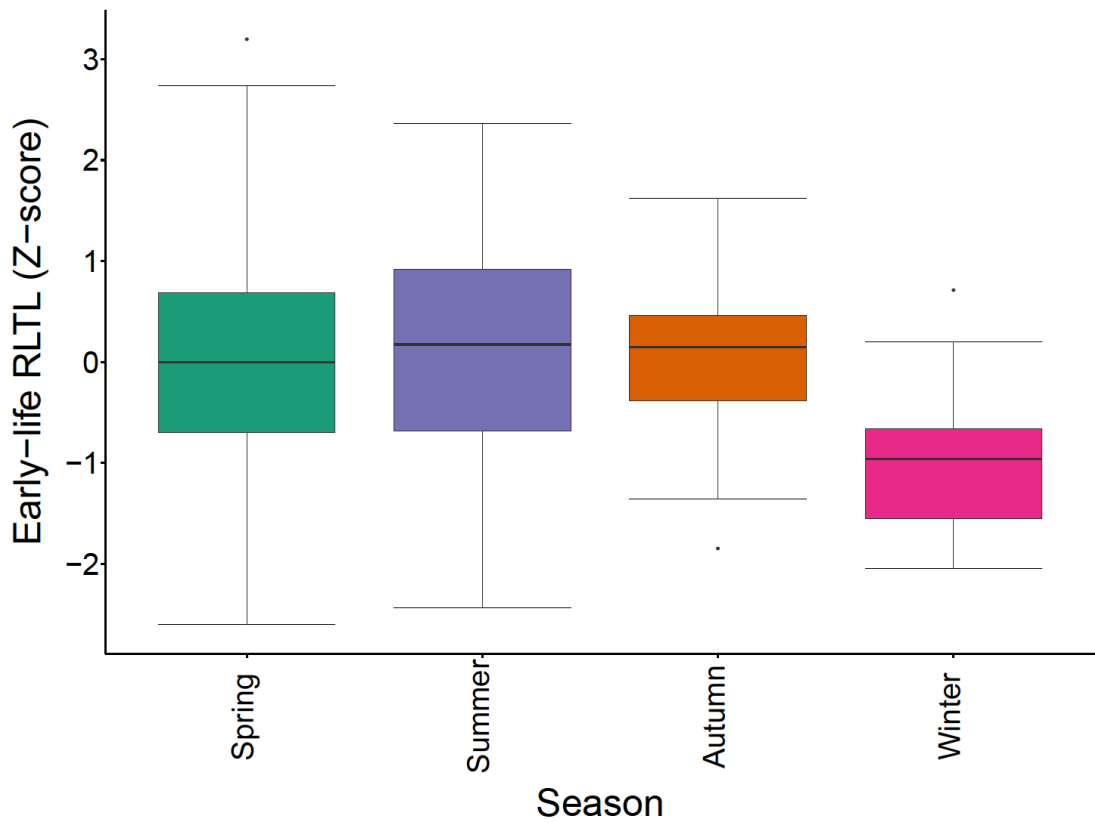
<b>Parameter (reference level)</b>	<b><math>\beta</math></b>	<b>S.E.</b>	<b>95% CI</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Intercept	-0.051	0.114	-0.219 to 0.169		
<b>Number of cubs in natal group</b>	<b>0.109</b>	<b>0.049</b>	<b>0.014 to 0.202</b>	<b>5.111</b>	<b>0.024</b>
Sex (Female)	-0.026	0.088	-0.188 to 0.159	0.027	0.869
Number of adult females in natal group	-0.042	0.048	-0.134 to 0.060	0.654	0.419
<b>Season (Spring)</b>				<b>12.70</b>	<b>0.005</b>
<b>Summer</b>	<b>0.096</b>	<b>0.105</b>	<b>-0.123 to 0.288</b>		
<b>Autumn</b>	<b>-0.039</b>	<b>0.189</b>	<b>-0.459 to 0.278</b>		
<b>Winter</b>	<b>-0.827</b>	<b>0.269</b>	<b>-1.418 to -0.377</b>		
<b>Mean temperature</b>	<b>0.250</b>	<b>0.078</b>	<b>0.138 to 0.377</b>	<b>11.75</b>	<b>&lt;0.001</b>
<b>Daily temperature variability</b>	<b>0.129</b>	<b>0.080</b>	<b>0.010 to 0.256</b>	<b>4.357</b>	<b>0.037</b>
<b>Mean daily rainfall</b>	<b>0.256</b>	<b>0.084</b>	<b>0.120 to 0.385</b>	<b>11.67</b>	<b>&lt;0.001</b>
<b>Coefficient of variation in daily rainfall</b>	<b>0.182</b>	<b>0.082</b>	<b>0.061 to 0.316</b>	<b>7.146</b>	<b>0.008</b>
<b>Mean temperature * Daily temperature variability</b>	<b>0.258</b>	<b>0.065</b>	<b>0.168 to 0.382</b>	<b>14.94</b>	<b>&lt;0.001</b>
<b>Mean daily rainfall * Coefficient of variation in daily rainfall</b>	<b>-0.355</b>	<b>0.113</b>	<b>-0.557 to -0.188</b>	<b>11.91</b>	<b>&lt;0.001</b>

Random effect estimates (variance): Plate ( $4.659 \times 10^{-2}$ ), Row ( $6.313 \times 10^{-3}$ ), Natal social group ( $9.930 \times 10^{-3}$ ), Cohort ( $5.734 \times 10^{-2}$ ), Residual ( $7.714 \times 10^{-1}$ )

**Table S4.4: Parameter estimates and 95% confidence intervals of fixed effects from a mixed model testing same-sex group size effects on relative leukocyte telomere length in early adulthood (Z-score) in European badgers.  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval,  $\chi^2$  = chi-squared value with associated p-value; reference terms in brackets = reference level for factors; \* = interaction. Significant parameters ( $p < 0.05$ ) are in bold.**

<b>Parameter (reference level)</b>	<b><math>\beta</math></b>	<b>S.E.</b>	<b>95% CI</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
Intercept	-0.094	0.280	-0.639 to 0.445		
Number of same-sex adults in group	-0.081	0.289	-0.648 to 0.476	0.151	0.770
Sex (Female)	0.185	0.276	-0.345 to 0.729	0.476	0.490
Season (Spring)				0.769	0.857
Summer	-0.055	0.111	-0.274 to 0.157		
Autumn	0.117	0.200	-0.276 to 0.505		
Winter	-0.040	0.305	-0.634 to 0.552		
Age at last capture	0.043	0.052	-0.058 to 0.143	0.693	0.405
Number of same-sex adults in group * Sex (Female)	0.034	0.296	-0.539 to 0.614	0.016	0.898

Random effect estimates (variance): Plate ( $4.315 \cdot 10^{-2}$ ), Row ( $<1.000 \cdot 10^{-12}$ ), Social group ( $8.914 \cdot 10^{-9}$ ), Cohort ( $2.297 \cdot 10^{-2}$ ), Year ( $1.721 \cdot 10^{-1}$ ), Individual ID ( $2.359 \cdot 10^{-8}$ ), Residual ( $8.397 \cdot 10^{-1}$ )



**Figure S4.1: Variation in early-life relative leukocyte telomere length (RLTL) among seasons in European badgers. Raw data are shown where the line represents the median, with first and third quartile, and whiskers represent 1.57 times the inter-quartile range.**

## **Chapter 5: Estimation of environmental and genetic contributions to telomere length variation in a wild mammal**

## 5.1 Abstract

Understanding individual variation in senescence patterns requires separating the environmental and genetic determinants. Telomeres are protective caps at the ends of chromosomes that are a biomarker of senescence as their length predicts mortality risk. The relative contribution of genetic and environmental factors to individual variation in telomere length is however unclear, yet important to understand its evolutionary dynamics. In particular, the evidence for transgenerational effects, in terms of parental age at conception, on telomere length is mixed. Here, we investigate the heritability of telomere length, using the ‘animal model’, and parental age at conception effects on offspring telomere length in a wild population of European badgers (*Meles meles*). While we found no heritability of telomere length, our power to detect heritability was low, and a repeatability of 2% across individual lifetimes provides a low upper-limit to ordinary heritability. However, year (25%) and cohort (3%) explained greater proportions of the phenotypic variance in telomere length. There was no support for parental age at conception effects, or for longitudinal within-parental age effects on offspring telomere length. Our results indicate a lack of transgenerational effects through parental age at conception effects and a low potential for evolutionary change in telomere length. Instead, we provide evidence that variation in environmental factors drives individual variation in telomere length in this wild mammal.

## 5.2 Introduction

Senescence is the progressive accumulation of damage with age (Medawar, 1952; Williams, 1957), where senescent declines in both cellular and physiological functions lead to age-dependent declines in survival probability and reproductive success (Stearns, 1992; Kirkwood and Austad, 2000). Evolutionary theories of senescence have been developed based on the premise that deleterious mutations or antagonistically pleiotropic genes put greater pressure on individual fitness in early-life compared to later-life (Medawar, 1952;

Williams, 1957; Kirkwood and Rose, 1991). Such pressure on fitness in early-life is the consequence of higher extrinsic mortality, leading to weakening forces of selection with age (Hamilton, 1966). These forces of natural selection, acting on the heritability of a trait (the proportion of phenotypic variance explained by additive genetic variance), can describe the evolutionary potential of senescence (Lynch and Walsh, 1998; Charmantier et al., 2014). It is therefore important to separate environmental and genetic components that contribute to individual variation in senescence patterns in order to understand the evolution of senescence (Charmantier et al., 2014; Wilson et al., 2008; Nussey et al., 2013).

Telomeres are a biomarker of senescence (Monaghan and Haussmann, 2006) and understanding the heritability of telomere length provides insight into the evolution of senescence (Dugdale and Richardson, 2018). Telomeres are repetitive non-coding sequences (5'-TTAGGG-3') at the ends of eukaryotic chromosomes that, along with shelterin proteins, maintain genomic integrity and prevent end-to-end fusion of linear chromosomes (Blackburn, 1991). Due to the end-replication problem, telomeres shorten with each cell division (Olovnikov, 1973). Telomere shortening can, however, be accelerated by adverse environmental conditions (e.g. Boonekamp et al., 2014; Nettle et al., 2015) and metabolically demanding activities (Heidinger et al., 2012; Epel et al., 2004). *In vitro* evidence shows that oxidative damage contributes to telomere shortening (von Zglinicki, 2002), but there is no evidence for such effects *in vivo* (Reichert and Stier, 2017; Boonekamp, 2017). Telomeres can also be restored by telomerase, although this enzyme is transcriptionally repressed after initial development (Blackburn et al., 1989). However, alternative telomere lengthening pathways exist (Cesare and Reddel, 2010; Mendez-Bermudez et al., 2012). Critically short telomeres can result in replicative senescence, where accumulation of senescent cells can impair tissue functioning (Armanios and Blackburn, 2012; Campisi, 2005) and may lead to organismal senescence (Young, 2018).

Individual variation in telomere length occurs in wild populations (Fairlie et al., 2016; Spurgin et al., 2017; van Lieshout et al., 2019) which is linked to individual life-history (Wilbourn et al., 2018). Understanding the degree to which individual variation in telomere length is due to genetic and environmental effects, in addition to the strength of natural selection acting on telomere length, allows estimation of the potential for evolutionary change (Lynch and Walsh, 1998; Charmantier et al., 2014). Heritability of telomere length has been estimated in over seven wild species and in >26 studies in humans (see Table 1 in Dugdale and Richardson, 2018). These studies primarily used parent–offspring regressions to determine the heritability of telomere length, with estimates ranging from 0 to 1. The majority, however, of these heritability estimates were relatively high, which is unexpected given that heritabilities of traits closely related to fitness are often low (Price and Schluter, 1991; Postma, 2014; Mousseau and Roff, 1987). However, parents and offspring often live in similar environments, and parent–offspring regressions are frequently confounded by these ‘shared environment’ effects, which can inflate heritability estimates (Kruuk, 2004).

The ‘animal model’ provides a statistical approach that can overcome the drawbacks of parent–offspring regressions because it allows partitioning of variance components into additive genetic and shared environment sources (Kruuk and Hadfield, 2007; Wilson et al., 2010). Because heritability is the proportion of phenotypic variation due to additive genetic variance, any changes to the amount of environmental variation will impact heritability estimates, even if the additive genetic variance does not itself change (Kruuk and Hadfield, 2007; Dugdale and Richardson, 2018). Environmental effects (e.g. Boonekamp et al., 2014; Nettle et al., 2015) therefore need to be accounted for to derive accurate heritability estimates (Dugdale and Richardson, 2018). The ‘animal model’ is a mixed-effects model that uses either the expected proportion of the genome that individuals share by descent (from a pedigree) or by state (from genomic data) to partition phenotypic variance into environmental and genetic components (Wilson et al., 2010). The few studies applying an



animal model approach in wild populations of non-human vertebrates found no heritability of telomere length in white-throated dippers (*Cinclus cinclus*;  $0.007 \pm 0.013$  SE; Becker et al., 2015), but high heritability in great reed warblers (*Acrocephalus arundinaceus*;  $0.480 \pm 0.120$  SE; Asghar et al., 2015a). However, although these were pioneering studies, the sample sizes were relatively low for quantitative genetic analyses and the power to detect heritability was not stated. Additionally, neither study had repeated measures to estimate permanent environment effects, which may inflate additive genetic effects (Kruuk and Hadfield, 2007). More studies in wild populations, and from more taxa, with larger sample sizes and repeated measures, are required to disentangle the genetic and environmental contributions to variation in telomere length.

The influence of environmental conditions on variation in telomere length is not only important to account for statistically, but informs about which environmental factors shape individual telomere length. Previous studies have shown that cohort (Hall et al., 2004; Watson et al., 2015; Fairlie et al., 2016), year (Mizutani et al., 2013; Wilbourn et al., 2017), social group (Cram et al., 2017; Boonekamp et al., 2014; Nettle et al., 2015) and parental effects (Asghar et al., 2015a; Cram et al., 2017) affect individual telomere length. Understanding the relative contribution of these different sources of environmental variation on telomere length informs on its evolution.

In addition to these environmental and additive genetic effects, offspring telomere length may also be influenced by paternal age at conception (PAC) according to two mutually non-exclusive hypotheses. First, to compensate for telomere loss due to sperm production and progressive cell replication, telomerase activity in germ stem cells is high. Telomerase expression might, beyond restoring telomere length, overcompensate and result in elongation of telomeres in germ stem cells (Kimura et al., 2008; Aviv and Susser, 2013). Second, stem cells with longer telomeres are better able to withstand repeated cell replication and therefore may become predominant in the stem cell pool with age due to the

selective loss of germ stem cells with shorter telomeres (Kimura et al., 2008; Hjelmborg et al., 2015). In humans, there is cross-sectional evidence that older men produce sperm with longer telomeres ( $r = 0.127-0.160$ ; Aston et al., 2012; de Meyer et al., 2007; Kimura et al., 2008; Nordfjall et al., 2010).

The evidence for a positive cross-sectional PAC effect is even stronger in captive chimpanzees (*Pan troglodytes*;  $r = 0.378$ ) compared to humans (Eisenberg et al., 2017). An explanation for this stronger effect is that chimpanzees have larger testes and higher rates of sperm production than humans, due to their more promiscuous mating system (Birkhead and Møller, 1998). Stronger sperm competition could therefore result in the PAC effect, because stronger postcopulatory competition should select for high quality sperm to be produced at a fast rate (Eisenberg et al., 2017). We would therefore expect that species with high levels of sperm competition and high rates of sperm production, such as in polygynandrous species, should show the strongest PAC effect.

PAC effects are often confounded with maternal age at conception (MAC), as these are typically highly correlated in human populations (Table 1 in Froy et al., 2017). The presence of MAC effects in humans is generally considered to be due to the correlation with PAC instead of a true independent biological effect (de Meyer et al., 2007; Kimura et al., 2008), because oocytes are produced prenatally, while sperm is produced throughout life (Eisenberg and Kuzawa, 2018). Additionally, parental age effects on fitness may be sex-specific (Bouwhuis et al., 2015). For example, male sparrows with older fathers or females with older mothers had lower lifetime reproductive success than sparrows with younger same-sex parents, and a hypothesised potential mechanism is sex-specific telomere shortening (Schroeder et al., 2015), although this was not the case in common terns (Bouwhuis et al., 2018). It is therefore important to test for sex-specific parental age effects.

Studies in wild populations have provided mixed evidence for PAC and MAC effects. Studies from different taxa, with a variety of mating systems, have shown a negative PAC

effect (Bouwhuis et al., 2018; Criscuolo et al., 2017; Olsson et al., 2011), including a longitudinal (Bauch et al., 2019) and an experimental manipulation (Noguera et al., 2018) study. However, other studies have reported no PAC or MAC effect on offspring telomere length (Heidinger et al., 2016; McLennan et al., 2018; Froy et al., 2017; Belmaker et al., 2019) or a positive MAC effect (Asghar et al., 2015a). The variation in PAC and MAC effects on offspring telomere length among species requires more studies to disentangle potential causes and mechanisms underlying such variation in transgenerational effects.

Here, we investigate PAC and MAC effects and the heritability of telomere length in polygynandrous European badgers (*Meles meles*; henceforth 'badgers'). Individual variation in badger telomere length in early-life (<1 year old) is associated with survival probability (van Lieshout et al., 2019). However, little heritability is expected, as within-individual repeatability in telomere length is very low (0.022, 95% CI = 0.001 – 0.103; van Lieshout et al., 2019). While this sets the upper limit for ordinary heritability (Bijma, 2011), understanding the relative importance of environmental (i.e. cohort, year, social group, maternal and paternal effects) and genetic variance components is important to understand the evolution of telomere length. Badgers respond to year-specific weather variation with effects on behaviour and fitness (Macdonald et al., 2010; Nouvellet et al., 2013; Noonan et al., 2014) and because they are group-living may be impacted by social group attributes (Woodroffe and Macdonald, 2000; Beirne et al., 2015). Cubs are born in February, which is followed by a post-partum mating peak after which matings can occur throughout the year (Macdonald et al., 2015). Badgers are highly promiscuous, which may promote sperm competition (Dugdale et al., 2011a). However, female badgers are induced ovulators thus requiring long matings (Yamaguchi et al., 2006), and associated testes ascendance in males in autumn/winter (Woodroffe and Macdonald, 1995a), lead to reduced sperm production rates (Sugianto et al., 2019b) that may reduce the potential for transgenerational effects (i.e. PAC/MAC effects) on offspring telomere length.

We therefore test for: (i) sex-specific and longitudinal PAC and MAC effects on offspring relative leukocyte telomere length (RLTL), after assessing whether PAC and MAC are correlated; and (ii) the proportion of variance in juvenile RLTL ( $\leq 29$  months old) and RLTL across individual lifetimes, that is explained by additive genetic and environmental effects.

## **5.3 Methods**

### **5.3.1 Study system**

We conducted this study in Wytham Woods, Oxfordshire, UK ( $51^{\circ}46'24''\text{N}$ ,  $1^{\circ}20'04''\text{W}$ ), a 424 ha mixed semi-natural woodland site surrounded by mixed arable and permanent pasture (Macdonald and Newman, 2002; Macdonald et al., 2004b). The resident badger population forms an almost closed population (immigration/emigration  $< 3\%$ ; Macdonald and Newman, 2002), with a mean number of 19 social groups (95% CI = 17–21; range = 14–26; Dugdale et al., 2008) between 1987–2010. Cohort-dependent cub survival probability varies from 0.61 to 0.94 (mean  $\pm$  SE =  $0.67 \pm 0.03$ ; Macdonald et al., 2009), whereas mean annual adult survival probability in the population is 0.83 ( $\pm 0.01$  SE; Macdonald et al., 2009) with a mean lifespan of 3.31 years ( $\pm 3.51$  SD; Bright Ross, J., Pers. Comm.).

Trappings were conducted three or four times per year over two weeks in May–June (Spring), August–September (Summer) and November (Autumn), with trapping in January (Winter) in focal years, for two to three consecutive days per social group. Trapped badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine hydrochloride per kg body weight (McLaren et al., 2005). Badgers were identified by a unique tattoo number on the left inguinal region. Sex, age class, sett (group den system), social group and capture date were recorded for each badger. Badgers were aged by the number of days elapsed since the 14<sup>th</sup> of February in the respective birth year (Yamaguchi et al., 2006). Individuals first caught as adults were aged through tooth wear, where tooth wear 2 indicates a 1-year old adult (van Lieshout et al., 2019). Blood was collected by jugular venipuncture into

vacutainers with an EDTA anticoagulant and stored at -20°C immediately. Badgers were released at their setts, after full recovery from anaesthesia.

### **5.3.2 Molecular analyses**

We extracted genomic DNA from whole blood samples ( $n = 1248$  samples; 612 badgers) using the DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol, with modifications by conducting a double elution step (2x 75  $\mu$ l AE buffer) and using 125  $\mu$ l of anticoagulated blood. We checked DNA integrity by running a random selection of DNA extracts (ca. 20%) on agarose gels to ensure high molecular weight. DNA concentration of all samples was quantified using the Fluostar Optima fluorometer (BMG Labtech, Ortenberg, Germany) and standardized to 20 ng/ $\mu$ l, after which samples were stored at -20 °C. We used monochrome multiplex quantitative PCR (MMqPCR) analysis to measure RLTL (Cawthon, 2009). This measure is the abundance of telomeric sequence relative to a reference gene, which are both analysed in the same well and represents the mean telomere length across cells in a sample. A detailed description of the MMqPCR analysis can be found in van Lieshout et al. (2019).

### **5.3.3 Pedigree**

The pedigree was constructed using DNA extracted from blood or guard hair samples, genotyped for 35 microsatellite loci (Dugdale et al., 2007; Annavi et al., 2014a), and *MasterBayes* 2.47 (Hadfield, 2010). The pruned pedigree (which excludes non-informative individuals) contained 753 unique individuals, from 7 generations, trapped between 1987 and 2010 (Table S5.1).

### **5.3.4 Statistical analyses**

*PAC and MAC effects*

Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2019). PAC and MAC effects were analysed in general linear mixed models (GLMMs), with RLTL measurements square-root transformed to meet assumptions of Gaussian error distributions and subsequently turned into Z-scores (Verhulst, 2019). We checked fixed effects for collinearity through variance inflation factors ( $VIF < 3$ ).

We first determined the correlation between PAC and MAC to investigate whether analyses for PAC and MAC effects needed to be conducted separately. There were 471 RLTL measurements from 240 offspring (121 females and 119 males; with 108 unique fathers and 120 unique mothers) where MAC and PAC were known. PAC and MAC both spanned ages 1–12 years and there was a weak positive correlation between PAC and MAC (Pearson's  $r = 0.160$ ,  $P < 0.001$ ; Figure S5.1), allowing for PAC and MAC effects to be tested in the same model.

The effects of PAC and MAC on offspring RLTL were subsequently tested using linear mixed effect models in *lme4* 1.1–14 (Bates et al., 2015). The model included fixed covariates for the best-fitting age relationship with RLTL, which was a threshold model (van Lieshout et al., 2019), and a fixed factor for season. Individual ID, cohort, year, qPCR plate, row on qPCR plate, maternal ID, paternal ID and social group were included as random effects. MAC and PAC were added to this model as fixed effects, and their interaction with sex, where significance was tested using likelihood ratio tests ( $n = 471$  measurements; 240 badgers). We have 80% statistical power, based on our dataset and model structure, to detect a PAC effect of 0.00067 or greater (Figure S5.2) using a simulation-based power analysis in *simr* 1.0.5 (Green and MacLeod, 2016). This is equivalent to a correlation coefficient of 0.131 or greater (with the PAC effect size multiplied by its standard deviation and divided by the standard deviation of RLTL), providing statistical power to detect correlation coefficients found previously in humans ( $r = 0.127$ – $0.160$ ; de Meyer et al., 2007; Eisenberg et al., 2017; Nordfjall et al., 2010) and chimpanzees ( $r = 0.378$ ; Eisenberg et al., 2017). Additional models were run,

where only offspring RLTL measurements from cubs (<1 year old) were included, to ensure the inclusion of adults did not mask effects of PAC or MAC. There were 194 measurements from 194 cubs (94 females, 100 males) that had 97 unique fathers and 109 unique mothers. The cub model was similar to the full model, but did not include random effects for individual ID (i.e. no repeat measures) and year (i.e. same as cohort random effect). We then separated, including all offspring RLTL measurements, within- from between-parental effects ( $n = 441$  measurements; 210 badgers) for each parent to test for longitudinal PAC and MAC effects, by taking the mean age at reproduction for each parent (between-parent effect) and subtracting this mean from each of the ages at reproduction of the parent (within-parent effect; van de Pol and Wright, 2009).

#### *Partitioning variance in RLTL*

We determined the relative contribution of environmental and genetic components to variation in RLTL with a quantitative genetic 'animal model' using pedigree relatedness based on parent-offspring assignments ( $n = 1248$  measurements; 612 badgers). We had 80% power to detect a heritability of RLTL of  $\geq 0.27$  (Figure S5.3), estimated using *pedantics* 1.7 (Morrissey and Wilson, 2010). We used a stepwise addition approach to facilitate the detection of confounding random effects (Charmantier et al., 2014), while estimating the changes in heritability in response to addition of random effects. Additionally, we present results without fixed effects, as random effects are conditioned on the fixed effects (Wilson, 2008). We used *MCMCglmm* 2.25 (Hadfield 2010), with the number of iterations set to 600,000, a thinning of 300 and burn-in period of 15,000 iterations. The response variable was untransformed RLTL to gain variance estimates on the scale the trait was measured on (de Villemereuil et al., 2016); only a square-root transformation of RLTL met Gaussian assumptions, however, a square-root link is not available in *MCMCglmm*. Three thresholds of age at measurement (van Lieshout et al., 2019) were included as fixed covariates and

season as a fixed factor. The random effects included: additive genetic, permanent environment (to account for environmental and non-additive genetic between-individual variation), parental effects (mother and father ID), year effects (cohort and capture years), resident social group, and measurement effects (qPCR-plate and row, to account for variance generated during the laboratory analysis). We present results with qPCR-plate and row included and excluded from the total phenotypic variance when calculating heritability, since qPCR-plate and row represent technical, not biological, variance (de Villemereuil et al., 2018).

Since badgers exhibit increases as well as decreases in RLTL in later life, and juvenile RLTL ( $\leq 29$  months old) does not vary with age cross-sectionally (van Lieshout et al., 2019), we also estimated variance components and heritability just using a dataset of juvenile RLTL ( $\leq 29$  months old;  $n = 837$  measurements; 556 badgers). We had 80% power to detect a heritability of  $\geq 0.28$  (Figure S5.4). The random effects were the same as in the full dataset. For the fixed effects the difference was that age was included as a linear covariate rather than a threshold model (as the first threshold is at 29 months; van Lieshout et al., 2019).

For random effects we used parameter expanded priors (F distribution:  $V = 1$ ,  $\nu = 1$ ,  $\alpha \cdot \mu = 0$ ,  $\alpha \cdot V = 1,000$ ) since variance components were close to zero. Model convergence was checked through low autocorrelation between successive thinned samples ( $< 0.1$ ), Heidelberg and Welch's diagnostics (tests if samples are drawn from stationary distribution), Geweke diagnostic (equality of means of first 10% and last 50% of Markov chain), and whether the effective size was  $> 1000$  for both fixed and variance components. Fixed effects were considered significant if the 95% credibility intervals of the posterior mode did not overlap zero.

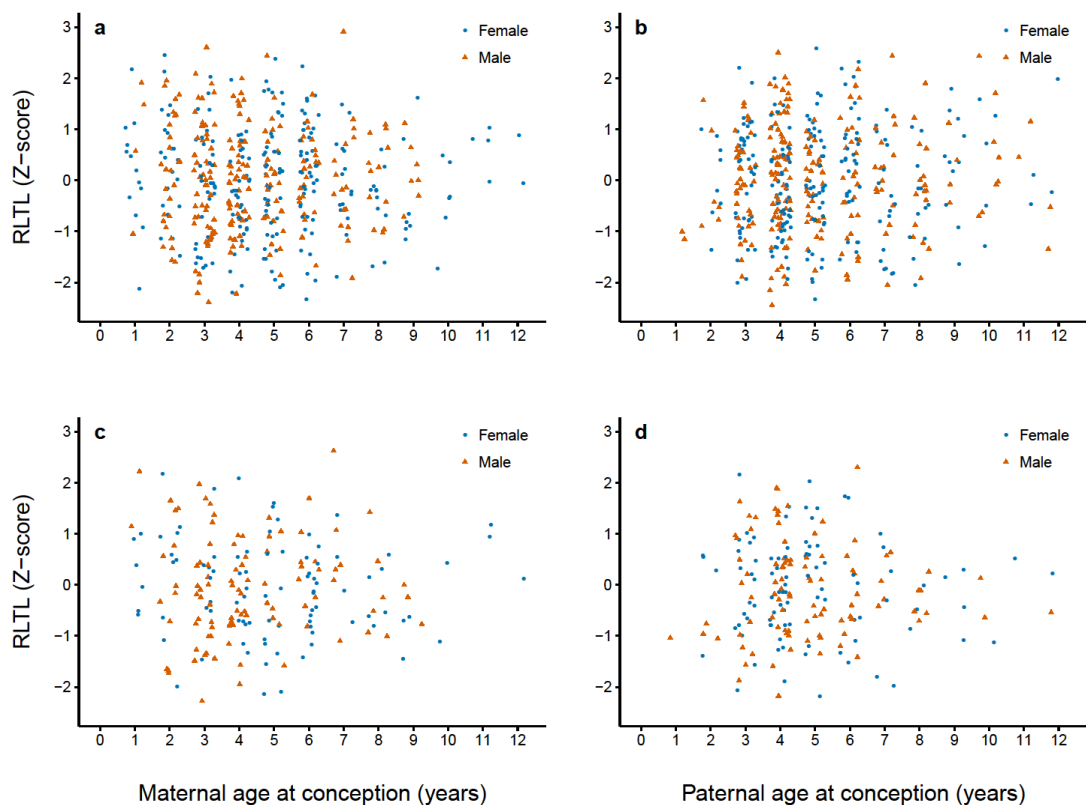
We also conducted a frequentist analysis in *ASReml-R* 3 using the same model structure to determine the robustness of our variance component estimates. In *ASReml-R*, the significance of fixed effects was determined through Wald Z tests, whereas significance



of random effects was determined through twice the difference in log-likelihood (Visscher, 2006).

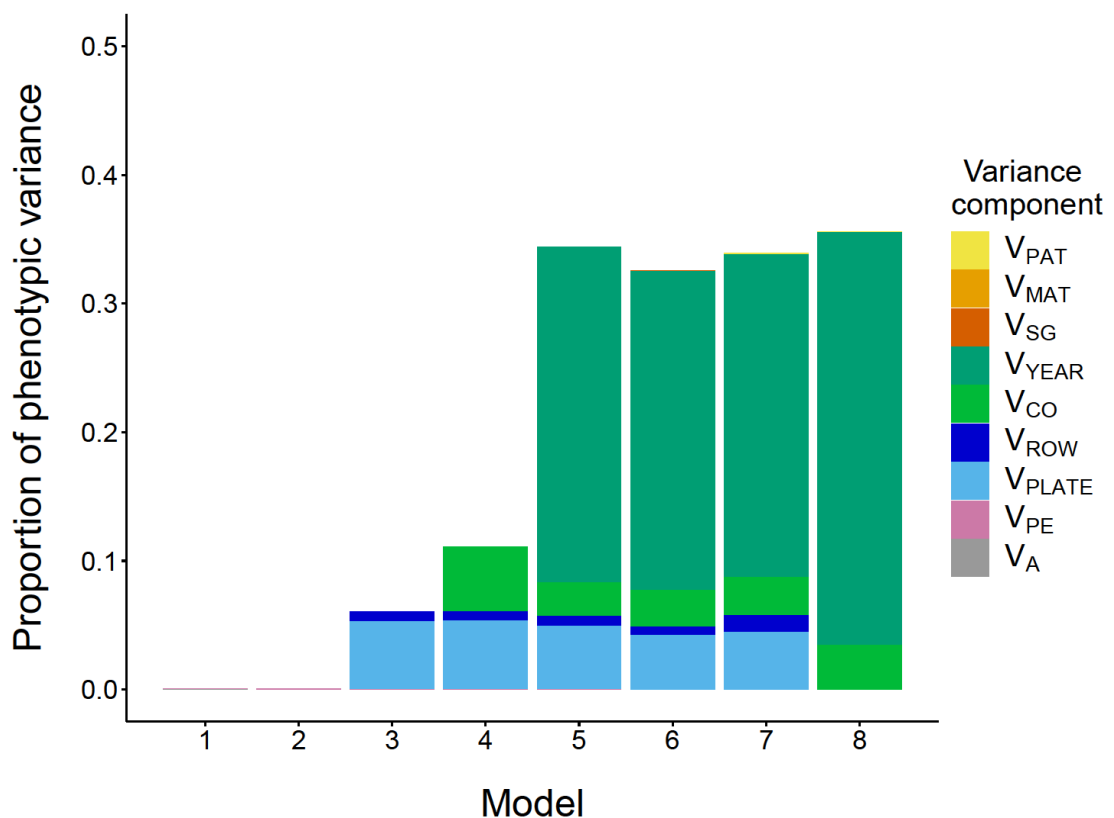
## 5.4 Results

Neither MAC nor PAC showed an overall or sex-specific association with variation in offspring RLTL at any age (Figure 5.1a & 5.1b, respectively), or as cubs (Figure 5.1c & 5.1d, respectively; Table S5.2). Additionally, within- and between-parental age at conception effects for each parent were not linked to variation in offspring RLTL (Table S5.2).



**Figure 5.1: Associations between offspring relative leukocyte telomere length (RLTL) and either maternal (a & c) or paternal (b & d) age at conception (years) in European badgers. Scatterplots show raw data (blue for females and brown for males) for all ages (a & b;  $n = 417$  measurements; 240 badgers) or only offspring measured as cubs (<1 year; c & d; 194 measurements; 194 badgers), and jittered for clarity.**

The additive genetic variance explained near zero of the total phenotypic variance in RLTL (Table S5.3, Models 5.1–5.9). Heritability ( $h^2$ ) was  $< 0.001$  (95% CrI =  $<0.001$ – $0.026$ ) with qPCR plate and row variance included in the phenotypic variance (Table S5.3, Model 5.7) and  $0.001$  (95% CrI =  $<0.001$ – $0.028$ ) when qPCR plate and row variance were excluded (Table S5.3, Model 5.8). In contrast, year ( $0.251$ , 95% CrI =  $0.143$ – $0.459$ ) and cohort ( $0.030$ , 95% CrI =  $0.007$ – $0.074$ ) explained a greater proportion of the phenotypic variance in RLTL (Figure 5.2; Table S5.3, Model 5.7). Social group ( $<0.001$ , 95% CrI =  $<0.001$ – $0.014$ ), paternal ( $<0.001$ , 95% CrI =  $<0.001$ – $0.025$ ) and maternal ( $<0.001$ , 95% CrI =  $<0.001$ – $0.030$ ) effects explained near zero variance in RLTL (Figure 5.2; Table S5.3, Model 5.7).



**Figure 5.2: Proportion of variance explained in relative leukocyte telomere length (RLTL; models 1–8) in European badgers. Variance components:  $V_A$  = additive genetic,  $V_{PE}$  = permanent environment,  $V_{PLATE}$  = plate,  $V_{ROW}$  = row,  $V_{CO}$  = cohort,  $V_{YEAR}$  = year,  $V_{SG}$  = social group,  $V_{MAT}$  = maternal, and  $V_{PAT}$  = paternal. Model numbers on the x-axis correspond with Table S5.3.**

There was also no detectable heritability of juvenile RLTL ( $\leq 29$  months old;  $h^2 < 0.001$ , 95% CrI =  $< 0.001$ – $0.043$ ), moderate year (0.216, 95% CrI =  $0.107$ – $0.431$ ) and cohort (0.037, 95% CrI =  $0.003$ – $0.123$ ) effects, and no detectable social group ( $< 0.001$ , 95% CrI =  $< 0.001$ – $0.020$ ), paternal ( $< 0.001$ , 95% CrI =  $< 0.001$ – $0.026$ ) or maternal ( $< 0.001$ , 95% CrI =  $< 0.001$ – $0.032$ ) effects (Table S5.3, Model 5.9).

A frequentist approach in *ASReml-R* showed similar results with additive genetic variance explaining near zero of the phenotypic variance, but with cohort and year effects explaining variation in RLTL (Table S5.4 & S5.5).

## 5.5 Discussion

Our study found no evidence for PAC or MAC associations with offspring RLTL in the European badger. Within primates there is extensive evidence for a positive PAC effect (e.g. Njajou et al., 2007; Eisenberg et al., 2017; Kimura et al., 2008). However, previous studies in non-primate vertebrates have reported a variety of relationships between offspring telomere length and PAC or MAC (e.g. Olsson et al., 2011; Asghar et al., 2015a; Heidinger et al., 2016; McLennan et al., 2018; Bouwhuis et al., 2018). Studies in non-primate mammals are limited. The one study in Soay sheep, which also has a promiscuous mating system with likely stronger sperm competition than in badgers (Preston et al., 2003), also found no relationship between offspring RLTL (either measured across all ages or only as lambs) and PAC or MAC (Froy et al., 2017). Our results add to the growing literature of mixed PAC/MAC results in wild populations (Olsson et al., 2011; Heidinger et al., 2016; Eisenberg et al., 2017; Njajou et al., 2007; McLennan et al., 2018), despite positive PAC effects in humans and chimpanzees (Njajou et al., 2007; Eisenberg et al., 2017; Kimura et al., 2008). Variation in PAC and MAC effects among species may be due to differences in mating systems and associated sperm production rates (Bouwhuis et al., 2018). Additionally, variation may be present but masked by sex-specific effects, however, we tested for but did not detect these.

Counter to our expectation for a highly promiscuous species that exhibits multiple and repetitive mounting behaviour (Dugdale et al., 2007; Dugdale et al., 2011a), we found no PAC effect, for which there are several potential reasons. First, telomerase activity may be more tightly regulated, or even lower, in the germline in badgers. However, while we know telomerase activity varies among tissue types and species (Davis and Kipling, 2005; Gomes et al., 2011), we require a better understanding of telomerase activity in species with different mating systems to validate this hypothesis. Secondly, higher sperm competition may reduce the variability in RLTL among germ stem cells, negating selection for germ stem cells with longer telomeres at older ages and therefore longer offspring RLTL (Kimura et al., 2008). Thirdly, female badgers exhibit various postcopulatory mechanisms (i.e. embryonic diapause, superfetation, superfecundation). Even though replication is suppressed during embryonic diapause, maternal stress could impact offspring RLTL through glucocorticoids (Hausmann et al., 2012; Angelier et al., 2018; Yamaguchi et al., 2006). Additionally, superfetation could benefit the later fertilised egg through less exposure to maternal glucocorticoids. Maternal stress during pregnancy may therefore obscure the relationship between PAC or MAC and offspring RLTL. However, the effects of these postcopulatory mechanisms on PAC and MAC effects are difficult to quantify. Finally, badgers have a much lower life expectancy than humans and chimpanzees (Macdonald and Newman, 2002), as do Soay sheep (Froy et al., 2017). While reproductive senescence is observed in both sexes (Dugdale et al., 2011b), the effects of telomere elongation in sperm may not become apparent due to the shorter life expectancy, compared to humans and chimpanzees. Even though in male badgers the testes ascend in autumn with no spermatogenesis (Sugianto et al., 2019b), sperm production is likely highest in the peak mating season immediately after parturition (Macdonald et al., 2015). Despite the high potential for sperm competition in this species, seasonal mating peaks in badgers may explain the lack of a PAC effect through the

lack of continuity and rate of sperm production in badgers, as recently hypothesised in Bouwhuis et al. (2018).

While our study reveals no heritability of RLTL, we did not have the statistical power to detect heritability of RLTL  $<0.27$ . Given that variance in RLTL explained by individual was very low 2%, which forms the upper limit to ordinary heritability, the contribution of additive genetic variance to total phenotypic variance in RLTL in this wild mammal population is low. The low heritability of RLTL is consistent with low heritability of fitness-related traits in other species (Kruuk et al., 2000; Teplitsky et al., 2009). We have previously identified associations between early-life RLTL ( $<1$  year old) and survival probability in this species (van Lieshout et al., 2019), so selection may have eroded genetic variation underlying RLTL in this population (Price and Schluter, 1991; Postma, 2014; Mousseau and Roff, 1987).

Partitioning of variation in RLTL in badgers into genetic and environmental factors showed that variation in RLTL was driven by environmental variation. Of the environmental factors investigated, we found no evidence for social group, maternal or paternal effects explaining variation in RLTL. Even though nest or social group (Nettle et al., 2015; Boonekamp et al., 2014; Cram et al., 2017; Becker et al., 2015) and maternal effects (Asghar et al., 2015a) have been important for telomere length in other species, this is not the case for badgers. Badgers provide neonatal care up to independence at around 14–16 weeks (Fell et al., 2006; Dugdale et al., 2010), and we therefore cannot capture badgers until at least 3 months of age (Protection of Badgers Act, 1992). As the strength of maternal effects on offspring decline with the age of the offspring (Moore et al., 2019), maternal effects explaining variation in offspring RLTL will be more difficult to detect. In contrast, we found that variation in RLTL was explained by a small cohort and moderate year effect.

The small effect of cohort on RLTL is in accordance with previous studies in mammals and birds which had shorter telomeres, or accelerated telomere shortening, when subject to sub-optimal natal conditions (Hall et al., 2004; Nettle et al., 2015; Watson et al., 2015; Fairlie

et al., 2016). However, the variance explained by the year in which the individual was captured was about eight times greater than the cohort effect, even though we couldn't separate cohort and year effects for 163 badgers since they died as cubs. Although we cannot identify the specific drivers of the association between year and variation in RLTL, badgers are sensitive to annual weather variation (Nouvellet et al., 2013; Macdonald et al., 2010), which affects their food availability, can lead to elevated levels of oxidative stress (Bilham et al., 2018) and affects early-life telomere length (Chapter 4). Additionally, exposure to diseases may vary among years and could contribute to variation in RLTL (Newman et al., 2001; Sin et al., 2014). The variation in badger RLTL is therefore largely driven by non-additive genetic sources such as variation between cohorts and years.

## Supplemental information Chapter 5: Estimation of environmental and genetic contributions to telomere length variation in a wild mammal

**Table S5.1: Information from pruned pedigree of the Wytham badger population (1987–2010).**

<b>Relationship</b>	<b><i>n</i></b>	<b>Relationship</b>	<b><i>n</i></b>
Records	753	Paternal grandmothers	261
Max. pedigree depth	7	Paternal grandfathers	214
Maternities	486	Founders	206
Paternities	458	Mean maternal sibship size	2.48
Full sibs	194	Mean paternal sibship size	2.59
Maternal sibs	691	Non-zero F	29
Paternal sibs	880	F > 0.125	11
Maternal half sibs	497	Mean pairwise relatedness	0.007
Paternal half sibs	686	Pairwise relatedness $\geq 0.125$	0.023
Maternal grandmothers	196	Pairwise relatedness $\geq 0.25$	0.013
Maternal grandfathers	174	Pairwise relatedness $\geq 0.5$	0.004

**Table S5.2: Parameter estimates from mixed model testing paternal and maternal age at conception (PAC & MAC, respectively) effects on offspring relative leukocyte telomere length (Z-score) in European badgers.  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95 % CI = 95 % confidence interval,  $\beta_w$  = within-individual effect,  $\beta_B$  = between-individual effect,  $\chi^2$  = chi-squared value and associated p-value, reference terms in brackets = reference level for factors; \* = interaction. Significant parameters (p-value < 0.05) are in bold.**

Parameter	$\beta$	S.E.	95% CI	$\chi^2$	p-value
<b>PAC/MAC model (cubs + adults – n = 471 measurements; 240 badgers)<sup>†</sup></b>					
Intercept	-0.014	0.127	-0.258 to 0.228		
Age ( $\leq 29$ months)	-0.016	0.068	-0.162 to 0.120	0.115	0.735
<b>(&gt;29 and <math>\leq 65</math> months)</b>	<b>0.180</b>	<b>0.079</b>	<b>0.030 to 0.338</b>	<b>5.317</b>	<b>0.021</b>
<b>(&gt;65 and <math>\leq 112</math> months)</b>	<b>-0.176</b>	<b>0.071</b>	<b>-0.311 to -0.038</b>	<b>6.138</b>	<b>0.013</b>
<b>(&gt; 112 months)</b>	<b>0.125</b>	<b>0.055</b>	<b>0.018 to 0.231</b>	<b>5.119</b>	<b>0.024</b>
Sex (female)	-0.021	0.093	-0.201 to 0.169	0.023	0.879
Season (Spring)				0.738	0.864
Summer	0.091	0.105	-0.120 to 0.294		
Autumn	-0.017	0.191	-0.387 to 0.353		
Winter	0.015	0.025	-0.459 to 0.509		
PAC	0.046	0.066	-0.081 to 0.176	0.491	0.491
MAC	-0.031	0.060	-0.148 to 0.088	0.238	0.627
Sex (female) * PAC	-0.018	0.092	-0.197 to 0.162	0.037	0.848
Sex (female) * MAC	-0.007	0.096	-0.194 to 0.181	0.006	0.939
<b>Within/Between-individual PAC/MAC (cubs + adults – n = 441 measurements; 210 badgers)<sup>††</sup></b>					
Intercept	0.026	0.115	-0.199 to 0.248		
Age ( $\leq 29$ months)	-0.043	0.069	-0.188 to 0.092	0.506	0.477
<b>(&gt;29 and <math>\leq 65</math> months)</b>	<b>0.197</b>	<b>0.080</b>	<b>0.043 to 0.359</b>	<b>6.055</b>	<b>0.014</b>
<b>(&gt;65 and <math>\leq 112</math> months)</b>	<b>-0.181</b>	<b>0.072</b>	<b>-0.320 to -0.040</b>	<b>6.265</b>	<b>0.012</b>
<b>(&gt; 112 months)</b>	<b>0.125</b>	<b>0.056</b>	<b>0.014 to 0.235</b>	<b>4.797</b>	<b>0.029</b>
Season (Spring)				0.224	0.974
Summer	0.059	0.108	-0.165 to 0.269		
Autumn	0.024	0.196	-0.355 to 0.405		
Winter	-0.012	0.249	-0.485 to 0.482		
PAC ( $\beta_w$ )	0.042	0.049	-0.051 to 0.140	0.838	0.360
MAC ( $\beta_w$ )	0.001	0.049	-0.092 to 0.098	0.005	0.945
PAC ( $\beta_B$ )	0.023	0.048	-0.070 to 0.116	0.236	0.627
MAC ( $\beta_B$ )	-0.063	0.050	-0.160 to 0.034	1.602	0.206
<b>PAC/MAC model (cubs – 194 measurements; 194 badgers)<sup>†††</sup></b>					
Intercept	0.037	0.204	-0.360 to 0.432		
Age	0.056	0.213	-0.354 to 0.468	0.078	0.780
Sex (female)	-0.107	0.133	-0.365 to 0.145	0.694	0.405
Season (Spring)				5.021	0.170
Summer	0.064	0.278	-0.489 to 0.610		
Autumn	-0.845	0.649	-2.116 to 0.402		
Winter	-0.416	0.906	-2.167 to 1.306		
PAC	-0.040	0.091	-0.213 to 0.134	0.210	0.664
MAC	-0.075	0.084	-0.236 to 0.086	0.753	0.385
Sex (female) * PAC	0.183	0.130	-0.064 to 0.435	2.035	0.154



Sex (female) * MAC	0.015	0.136	-0.246 to 0.276	0.012	0.912
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Random effect estimates (variance): <sup>†</sup>Cohort ( $1.920 \times 10^{-2}$ ), Social group ( $2.116 \times 10^{-2}$ ), Year ( $5.795 \times 10^{-2}$ ), Plate ( $6.640 \times 10^{-2}$ ), Row ( $1.613 \times 10^{-2}$ ), individual ID ( $3.927 \times 10^{-8}$ ), mother ID ( $3.248 \times 10^{-10}$ ), father ID ( $1.340 \times 10^{-8}$ ), Residual ( $8.503 \times 10^{-1}$ ); <sup>††</sup>Cohort ( $8.221 \times 10^{-3}$ ), Social group ( $1.330 \times 10^{-2}$ ), Year ( $6.593 \times 10^{-2}$ ), Plate ( $6.992 \times 10^{-2}$ ), Row ( $1.538 \times 10^{-2}$ ), individual ID ( $1.368 \times 10^{-6}$ ), mother ID ( $<1.000 \times 10^{-12}$ ), father ID ( $<1.000 \times 10^{-12}$ ), Residual ( $8.537 \times 10^{-1}$ ); <sup>†††</sup>Cohort ( $8.221 \times 10^{-2}$ ), Social group ( $<1.000 \times 10^{-12}$ ), Plate ( $1.669 \times 10^{-1}$ ), Row ( $3.728 \times 10^{-2}$ ), mother ID ( $<1.000 \times 10^{-12}$ ), father ID ( $3.631 \times 10^{-3}$ ), Residual ( $6.391 \times 10^{-1}$ )

**Table S5.3: Additive genetic and environmental effects on relative leukocyte telomere length in European badgers, estimated using the ‘animal model’ with *MCMCglmm* 2.25 (Hadfield, 2010). Nine models are presented with random effects for additive genetic and permanent environment variance components. Subsequently, fixed and random effects are sequentially added to determine their effect on heritability. Values represent the posterior modes and 95% credible intervals of the variance estimates ( $V_A$  = additive genetic,  $V_{PE}$  = permanent environment,  $V_{PLATE}$  = plate,  $V_{ROW}$  = Row,  $V_{CO}$  = cohort,  $V_{YEAR}$  = year,  $V_{SG}$  = social group,  $V_{MAT}$  = maternal,  $V_{PAT}$  = paternal,  $V_R$  = residual,  $V_P$  = phenotypic). Age = threshold age,  $h^2$  = heritability.**

Model	Parameters	$V_A$	$V_{PE}$	$V_{PLATE}$	$V_{ROW}$	$V_{CO}$	$V_{YEAR}$	$V_{SG}$	$V_{MAT}$	$V_{PAT}$	$V_R$	$h^2$
5.1	TL = $V_A + V_{PE}$ ( $n = 1248$ )	1.51*10 <sup>-5</sup> (3.25*10 <sup>-9</sup> – 2.90*10 <sup>-3</sup> )	1.20*10 <sup>-5</sup> (1.73*10 <sup>-10</sup> – 2.25*10 <sup>-3</sup> )								3.28*10 <sup>-2</sup> (2.94*10 <sup>-2</sup> – 3.51*10 <sup>-2</sup> )	3.85*10 <sup>-4</sup> (9.00*10 <sup>-8</sup> – 8.44*10 <sup>-2</sup> )
5.2	TL = $V_A + V_{PE} + \text{Age} + \text{Season}$ ( $n = 1248$ )	1.99*10 <sup>-5</sup> (9.03*10 <sup>-11</sup> – –2.57*10 <sup>-3</sup> )	1.55*10 <sup>-5</sup> (1.39*10 <sup>-11</sup> – 2.14*10 <sup>-3</sup> )								3.17*10 <sup>-2</sup> (2.89*10 <sup>-2</sup> – 3.48*10 <sup>-2</sup> )	3.41*10 <sup>-4</sup> (2.97*10 <sup>-9</sup> – 7.60*10 <sup>-2</sup> )
5.3	TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW}$ ( $n = 1248$ )	1.37*10 <sup>-5</sup> (8.97*10 <sup>-11</sup> – –2.68*10 <sup>-3</sup> )	8.57*10 <sup>-6</sup> (4.24*10 <sup>-10</sup> – 2.16*10 <sup>-3</sup> )	1.84*10 <sup>-3</sup> (7.41*10 <sup>-4</sup> – 3.73*10 <sup>-3</sup> )	2.28*10 <sup>-4</sup> (2.17*10 <sup>-7</sup> – 2.09*10 <sup>-3</sup> )						2.96*10 <sup>-2</sup> (2.68*10 <sup>-2</sup> – 3.25*10 <sup>-2</sup> )	3.599*10 <sup>-4</sup> (2.64*10 <sup>-9</sup> – 7.94*10 <sup>-2</sup> )
5.4	TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW} + V_{CO}$ ( $n = 1248$ )	1.54*10 <sup>-5</sup> (2.22*10 <sup>-9</sup> – 1.82*10 <sup>-3</sup> )	6.13*10 <sup>-6</sup> (1.15*10 <sup>-9</sup> – 1.74*10 <sup>-3</sup> )	2.01*10 <sup>-3</sup> (7.81*10 <sup>-4</sup> – 3.68*10 <sup>-3</sup> )	2.54*10 <sup>-4</sup> (3.33*10 <sup>-14</sup> – –2.05*10 <sup>-3</sup> )	1.76*10 <sup>-3</sup> (7.54*10 <sup>-4</sup> – 5.31*10 <sup>-3</sup> )					2.90*10 <sup>-2</sup> (2.62*10 <sup>-2</sup> – 3.12*10 <sup>-2</sup> )	3.45*10 <sup>-4</sup> (5.72*10 <sup>-8</sup> – 5.10*10 <sup>-2</sup> )
5.5	TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW} + V_{CO} + V_{YEAR}$ ( $n = 1248$ )	8.04*10 <sup>-6</sup> (4.17*10 <sup>-12</sup> – –1.57*10 <sup>-3</sup> )	5.45*10 <sup>-6</sup> (1.35*10 <sup>-9</sup> – 1.53*10 <sup>-3</sup> )	1.90*10 <sup>-3</sup> (1.01*10 <sup>-3</sup> – 4.07*10 <sup>-3</sup> )	3.32*10 <sup>-4</sup> (6.15*10 <sup>-9</sup> – 2.37*10 <sup>-3</sup> )	1.15*10 <sup>-3</sup> (3.70*10 <sup>-4</sup> – 3.63*10 <sup>-3</sup> )	1.20*10 <sup>-2</sup> (4.35*10 <sup>-3</sup> – 2.47*10 <sup>-2</sup> )				2.57*10 <sup>-2</sup> (2.34*10 <sup>-2</sup> – 2.81*10 <sup>-2</sup> )	2.28*10 <sup>-4</sup> (8.82*10 <sup>-11</sup> – –3.44*10 <sup>-2</sup> )
5.6	TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW} + V_{CO} + V_{YEAR} + V_{SG}$ ( $n = 1248$ )	4.43*10 <sup>-6</sup> (4.07*10 <sup>-10</sup> – –1.46*10 <sup>-3</sup> )	8.68*10 <sup>-6</sup> (3.66*10 <sup>-10</sup> – 1.52*10 <sup>-3</sup> )	1.87*10 <sup>-3</sup> (9.78*10 <sup>-4</sup> – 4.13*10 <sup>-3</sup> )	3.10*10 <sup>-4</sup> (8.69*10 <sup>-9</sup> – 2.33*10 <sup>-3</sup> )	1.22*10 <sup>-3</sup> (4.25*10 <sup>-4</sup> – 3.65*10 <sup>-3</sup> )	1.03*10 <sup>-2</sup> (4.89*10 <sup>-3</sup> – 2.56*10 <sup>-2</sup> )	3.28*10 <sup>-6</sup> (1.56*10 <sup>-10</sup> – –7.13*10 <sup>-4</sup> )			2.55*10 <sup>-2</sup> (2.36*10 <sup>-2</sup> – 2.82*10 <sup>-2</sup> )	1.79*10 <sup>-4</sup> (1.12*10 <sup>-8</sup> – 3.21*10 <sup>-2</sup> )
5.7	TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW} + V_{CO} + V_{YEAR} + V_{SG} + V_{MAT} + V_{PAT}$ ( $n = 1248$ )	8.54*10 <sup>-6</sup> (1.01*10 <sup>-9</sup> – 1.19*10 <sup>-3</sup> )	8.64*10 <sup>-6</sup> (3.87*10 <sup>-10</sup> – 1.23*10 <sup>-3</sup> )	2.04*10 <sup>-3</sup> (9.68*10 <sup>-4</sup> – 4.12*10 <sup>-3</sup> )	5.15*10 <sup>-4</sup> (6.73*10 <sup>-7</sup> – 2.46*10 <sup>-3</sup> )	1.21*10 <sup>-3</sup> (2.86*10 <sup>-4</sup> – 3.36*10 <sup>-3</sup> )	1.16*10 <sup>-2</sup> (4.66*10 <sup>-3</sup> – 2.59*10 <sup>-2</sup> )	2.56*10 <sup>-6</sup> (3.78*10 <sup>-10</sup> – –6.40*10 <sup>-4</sup> )	1.12*10 <sup>-5</sup> (7.70*10 <sup>-12</sup> – –1.35*10 <sup>-3</sup> )	5.82*10 <sup>-6</sup> (6.90*10 <sup>-11</sup> – –1.11*10 <sup>-3</sup> )	2.53*10 <sup>-2</sup> (2.32*10 <sup>-2</sup> – 2.78*10 <sup>-2</sup> )	7.66*10 <sup>-5</sup> (2.03*10 <sup>-8</sup> – 2.58*10 <sup>-2</sup> )
5.8	TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW} + V_{CO} + V_{YEAR} + V_{SG} + V_{MAT} + V_{PAT}$ ( $n = 1248$ ); without $V_{PLATE}$ and $V_{ROW}$ in $V_P$	8.54*10 <sup>-6</sup> (1.01*10 <sup>-9</sup> – 1.19*10 <sup>-3</sup> )	8.64*10 <sup>-6</sup> (3.87*10 <sup>-10</sup> – 1.23*10 <sup>-3</sup> )	2.04*10 <sup>-3</sup> (9.68*10 <sup>-4</sup> – 4.12*10 <sup>-3</sup> )	5.15*10 <sup>-4</sup> (6.73*10 <sup>-7</sup> – 2.46*10 <sup>-3</sup> )	1.21*10 <sup>-3</sup> (2.86*10 <sup>-4</sup> – 3.36*10 <sup>-3</sup> )	1.16*10 <sup>-2</sup> (4.66*10 <sup>-3</sup> – 2.59*10 <sup>-2</sup> )	2.56*10 <sup>-6</sup> (3.78*10 <sup>-10</sup> – –6.40*10 <sup>-4</sup> )	1.120*10 <sup>-5</sup> (7.70*10 <sup>-12</sup> – –1.35*10 <sup>-3</sup> )	5.82*10 <sup>-6</sup> (6.90*10 <sup>-11</sup> – –1.11*10 <sup>-3</sup> )	2.53*10 <sup>-2</sup> (2.32*10 <sup>-2</sup> – 2.78*10 <sup>-2</sup> )	8.60*10 <sup>-5</sup> (2.17*10 <sup>-8</sup> – 2.76*10 <sup>-2</sup> )
5.9	Juvenile TL = $V_A + V_{PE} + \text{Age} + \text{Season} + V_{PLATE} + V_{ROW} + V_{CO} + V_{YEAR} + V_{SG} + V_{MAT} + V_{PAT}$ ( $n = 837$ )	1.27*10 <sup>-5</sup> (1.79*10 <sup>-9</sup> – 1.80*10 <sup>-3</sup> )	1.12*10 <sup>-5</sup> (1.31*10 <sup>-9</sup> – 2.12*10 <sup>-3</sup> )	1.57*10 <sup>-3</sup> (6.60*10 <sup>-4</sup> – 3.73*10 <sup>-3</sup> )	4.30*10 <sup>-5</sup> (9.76*10 <sup>-9</sup> – 1.44*10 <sup>-3</sup> )	1.77*10 <sup>-3</sup> (1.35*10 <sup>-4</sup> – 5.65*10 <sup>-3</sup> )	8.61*10 <sup>-3</sup> (3.17*10 <sup>-3</sup> – 2.30*10 <sup>-2</sup> )	3.140*10 <sup>-6</sup> (1.74*10 <sup>-9</sup> – 9.26*10 <sup>-4</sup> )	5.35*10 <sup>-6</sup> (3.91*10 <sup>-12</sup> – –1.40*10 <sup>-3</sup> )	5.81*10 <sup>-6</sup> (1.21*10 <sup>-9</sup> – 1.16*10 <sup>-3</sup> )	2.49*10 <sup>-2</sup> (2.28*10 <sup>-2</sup> – 2.89*10 <sup>-2</sup> )	2.49*10 <sup>-4</sup> (4.06*10 <sup>-8</sup> – 4.01*10 <sup>-2</sup> )

**Table S5.4: Additive genetic and environmental effects on relative leukocyte telomere length in European badgers, estimated using ASReml-R. Est. = fixed effect estimate, Prop. = proportion of variance explained, S.E. = Standard error,  $h^2$  = heritability. Age parameters refer to threshold model where Age 1 =  $\leq 29$  months old, Age 2 =  $> 29$  and  $\leq 65$  months old, Age 3 =  $> 65$  and  $\leq 112$  months old and Age 4 =  $> 112$  months old. Variance components are:  $V_A$  = additive genetic,  $V_{PE}$  = permanent environment,  $V_{CO}$  = cohort,  $V_{YEAR}$  = year,  $V_{SG}$  = social group,  $V_{MAT}$  = maternal,  $V_{PAT}$  = paternal,  $V_R$  = residual,  $V_{PLATE}$  = plate,  $V_{ROW}$  = row. Total phenotypic variance ( $V_p$ ) =  $3.970 \times 10^{-2}$ . Reference terms in brackets = reference level for factors. Significance of fixed effects was determined through Wald Z tests, and for random effects through twice the difference in log-likelihood. Significant parameters ( $p < 0.05$ ) are in bold.**

Trait: Relative leukocyte telomere length ( $n = 1248$ measurements; 612 badgers)				
ASReml-R				
	Est.	S.E.	F-value	p-value
<b>Fixed effects</b>				
Intercept	0.4796	$2.933 \times 10^{-2}$		
Age 1	$-2.888 \times 10^{-4}$	$6.920 \times 10^{-4}$	0.174	0.677
<b>Age 2</b>	<b><math>2.156 \times 10^{-3}</math></b>	<b><math>6.755 \times 10^{-4}</math></b>	<b>10.18</b>	<b>0.001</b>
<b>Age 3</b>	<b><math>-2.570 \times 10^{-3}</math></b>	<b><math>8.358 \times 10^{-4}</math></b>	<b>9.454</b>	<b>0.002</b>
<b>Age 4</b>	<b><math>4.766 \times 10^{-3}</math></b>	<b><math>2.036 \times 10^{-3}</math></b>	<b>5.481</b>	<b>0.019</b>
Season (Spring)			<b>3.627</b>	<b>0.013</b>
Summer	<b><math>2.448 \times 10^{-2}</math></b>	<b><math>1.123 \times 10^{-2}</math></b>		
Autumn	<b><math>1.841 \times 10^{-2}</math></b>	<b><math>1.799 \times 10^{-2}</math></b>		
Winter	<b><math>-5.365 \times 10^{-2}</math></b>	<b><math>2.521 \times 10^{-2}</math></b>		
<b>Random effects</b>	<b>Est. (S.E.)</b>	<b><math>h^2</math>/Prop. (S.E.)</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
$V_A^a$	$5.110 \times 10^{-9}$ ( $2.260 \times 10^{-10}$ )	$1.286 \times 10^{-7}$ ( $1.420 \times 10^{-8}$ )		
$V_{PE}^a$	$7.610 \times 10^{-9}$ ( $3.360 \times 10^{-10}$ )	$1.914 \times 10^{-7}$ ( $2.115 \times 10^{-8}$ )		
<b><math>V_{CO}</math></b>	<b><math>1.411 \times 10^{-3}</math> (<math>6.970 \times 10^{-4}</math>)</b>	<b><math>3.551 \times 10^{-2}</math> (<math>1.750 \times 10^{-2}</math>)</b>	<b>16.92</b>	<b>&lt;0.001</b>
<b><math>V_{YEAR}</math></b>	<b><math>1.161 \times 10^{-2}</math> (<math>4.230 \times 10^{-3}</math>)</b>	<b><math>0.292</math> (<math>7.632 \times 10^{-2}</math>)</b>	<b>79.94</b>	<b>&lt;0.001</b>
$V_{SG}$	$1.494 \times 10^{-4}$ ( $2.163 \times 10^{-4}$ )	$3.761 \times 10^{-3}$ ( $5.458 \times 10^{-3}$ )	0.700	0.403
$V_{MAT}$	$5.086 \times 10^{-4}$ ( $5.612 \times 10^{-4}$ )	$1.280 \times 10^{-2}$ ( $1.414 \times 10^{-2}$ )	0.544	0.461
$V_{PAT}$	$1.974 \times 10^{-4}$ ( $3.658 \times 10^{-4}$ )	$4.968 \times 10^{-3}$ ( $9.214 \times 10^{-3}$ )	0.412	0.521
$V_R$	$2.585 \times 10^{-2}$ ( $1.142 \times 10^{-3}$ )	$0.651$ ( $7.189 \times 10^{-2}$ )		
<b><math>V_{PLATE}^b</math></b>	<b><math>2.085 \times 10^{-3}</math> (<math>7.034 \times 10^{-4}</math>)</b>		<b>46.11</b>	<b>&lt;0.001</b>
<b><math>V_{ROW}^b</math></b>	<b><math>4.354 \times 10^{-4}</math> (<math>3.256 \times 10^{-4}</math>)</b>		<b>8.824</b>	<b>0.003</b>

<sup>a</sup> Significance not tested because variance components were at boundary

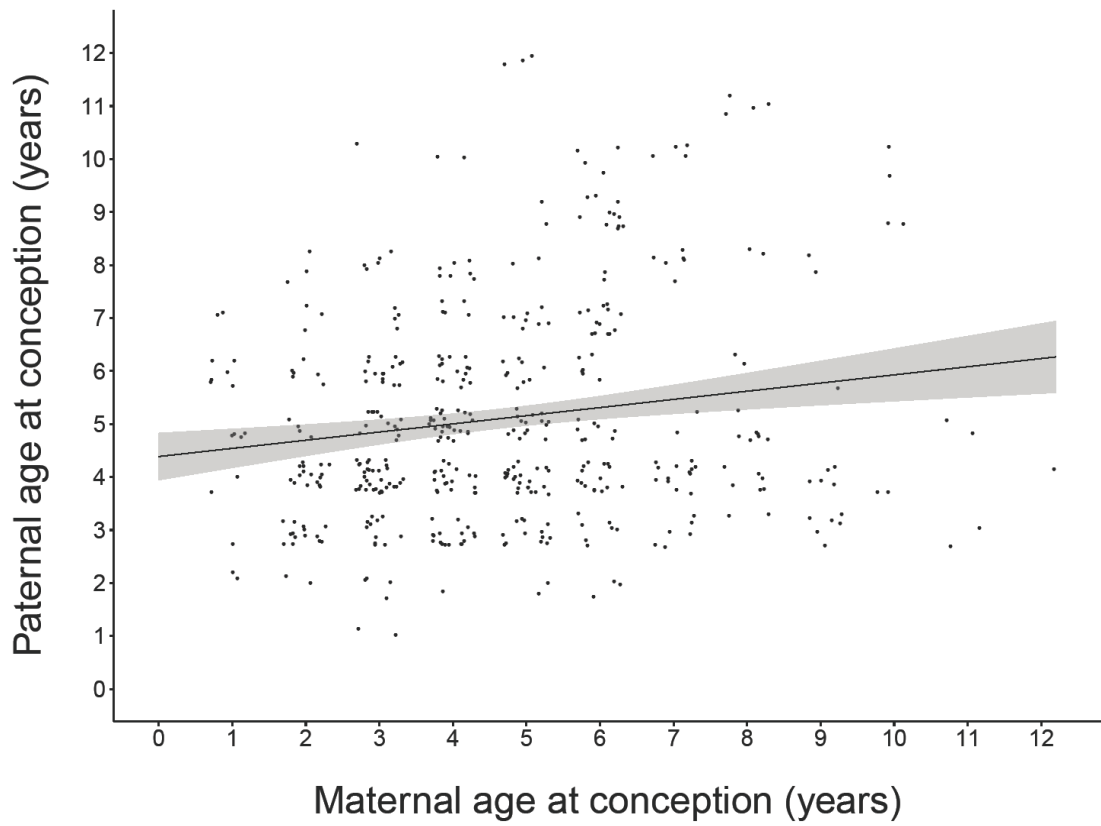
<sup>b</sup> Measurement error and not biological variance so no  $\chi^2$  and p-value provided

**Table S5.5: Additive genetic and environmental effects on relative leukocyte telomere length (Age ≤ 29 months) in European badgers, estimated using ASReml-R. Est. = fixed effect estimate, Prop. = proportion of variance explained, S.E. = Standard error,  $h^2$  = heritability. Variance components are:  $V_A$  = additive genetic,  $V_{PE}$  = permanent environment,  $V_{CO}$  = cohort,  $V_{YEAR}$  = year,  $V_{SG}$  = social group,  $V_{MAT}$  = maternal,  $V_{PAT}$  = paternal,  $V_R$  = residual,  $V_{PLATE}$  = plate,  $V_{ROW}$  = row. Total phenotypic variance ( $V_p$ ) =  $3.910 \times 10^{-2}$ . Reference terms in brackets = reference level for factors. Significance of fixed effects was determined through Wald Z tests, and for random effects through twice the difference in log-likelihood. Significant parameters ( $p < 0.05$ ) are in bold.**

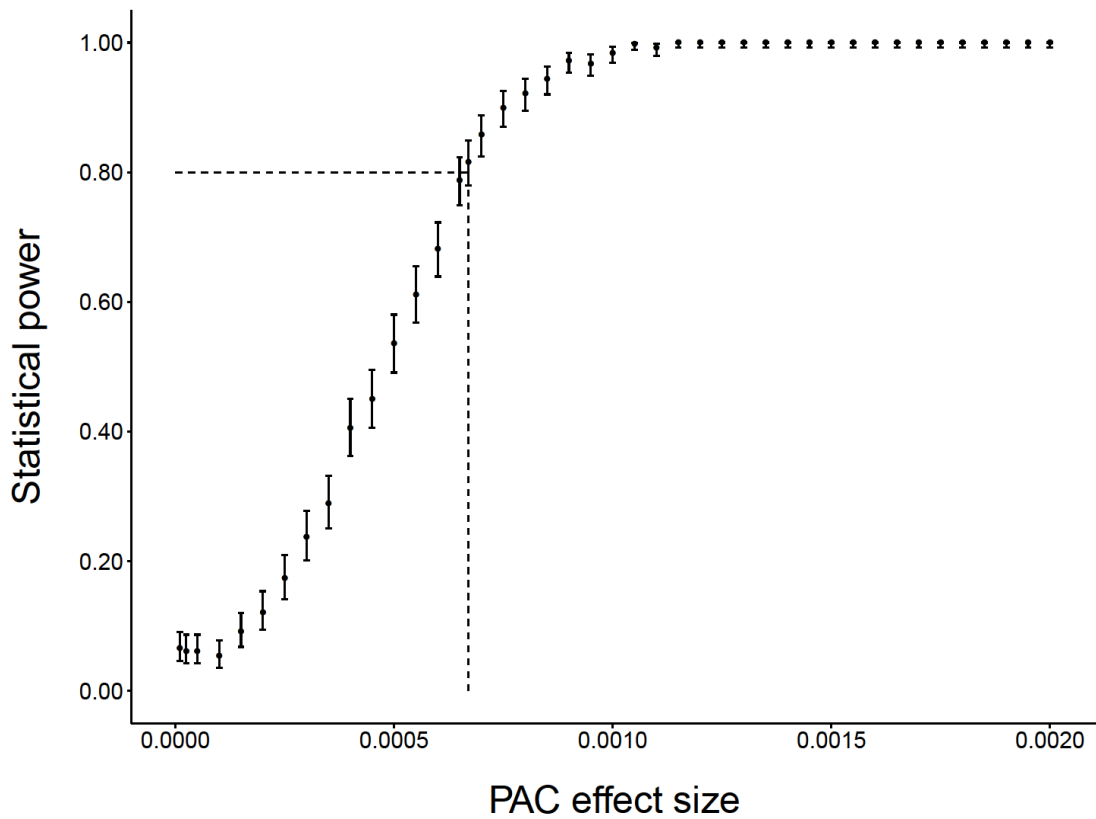
Trait: Early-life relative leukocyte telomere length ( $n = 837$ measurements; 556 badgers) ASReml-R				
	Est.	S.E.	F-value	p-value
<b>Fixed effects</b>				
Intercept	0.4771	$2.874 \times 10^{-2}$		
Age	$1.120 \times 10^{-4}$	$8.403 \times 10^{-4}$	0.018	0.894
<b>Season (Spring)</b>			<b>2.933</b>	<b>0.033</b>
<b>Summer</b>	<b><math>1.576 \times 10^{-2}</math></b>	<b><math>1.398 \times 10^{-2}</math></b>		
<b>Autumn</b>	<b><math>1.516 \times 10^{-2}</math></b>	<b><math>2.408 \times 10^{-2}</math></b>		
<b>Winter</b>	<b><math>-7.998 \times 10^{-2}</math></b>	<b><math>3.150 \times 10^{-2}</math></b>		
<b>Random effects</b>	<b>Est. (S.E.)</b>	<b><math>h^2</math>/Prop. (S.E.)</b>	<b><math>\chi^2</math></b>	<b>p-value</b>
$V_A$	$1.280 \times 10^{-4}$ ( $1.075 \times 10^{-3}$ )	$3.271 \times 10^{-3}$ ( $2.746 \times 10^{-2}$ )	0.014	0.906
$V_{PE}^a$	$8.770 \times 10^{-9}$ ( $5.560 \times 10^{-10}$ )	$2.240 \times 10^{-7}$ ( $2.569 \times 10^{-8}$ )		
<b><math>V_{CO}</math></b>	<b><math>1.945 \times 10^{-3}</math> (<math>1.138 \times 10^{-3}</math>)</b>	<b><math>4.970 \times 10^{-2}</math> (<math>2.862 \times 10^{-2}</math>)</b>	<b>11.13</b>	<b>&lt;0.001</b>
<b><math>V_{YEAR}</math></b>	<b><math>1.004 \times 10^{-2}</math> (<math>4.062 \times 10^{-3}</math>)</b>	<b><math>0.257</math> (<math>7.890 \times 10^{-2}</math>)</b>	<b>40.45</b>	<b>&lt;0.001</b>
$V_{SG}$	$2.412 \times 10^{-4}$ ( $3.169 \times 10^{-4}$ )	$6.162 \times 10^{-3}$ ( $8.107 \times 10^{-3}$ )	0.879	0.348
$V_{MAT}^a$	$2.710 \times 10^{-9}$ ( $1.720 \times 10^{-10}$ )	$6.924 \times 10^{-8}$ ( $7.940 \times 10^{-9}$ )		
$V_{PAT}^a$	$2.360 \times 10^{-9}$ ( $1.500 \times 10^{-10}$ )	$6.024 \times 10^{-8}$ ( $6.907 \times 10^{-9}$ )		
$V_R$	$2.679 \times 10^{-2}$ ( $1.699 \times 10^{-3}$ )	$0.684$ ( $7.846 \times 10^{-2}$ )		
<b><math>V_{PLATE}^b</math></b>	<b><math>1.771 \times 10^{-3}</math> (<math>7.239 \times 10^{-4}</math>)</b>		<b>19.49</b>	<b>&lt;0.001</b>
$V_{ROW}^b$	$1.942 \times 10^{-4}$ ( $2.461 \times 10^{-4}$ )		1.319	0.251

<sup>a</sup> Significance not tested because variance components were at boundary

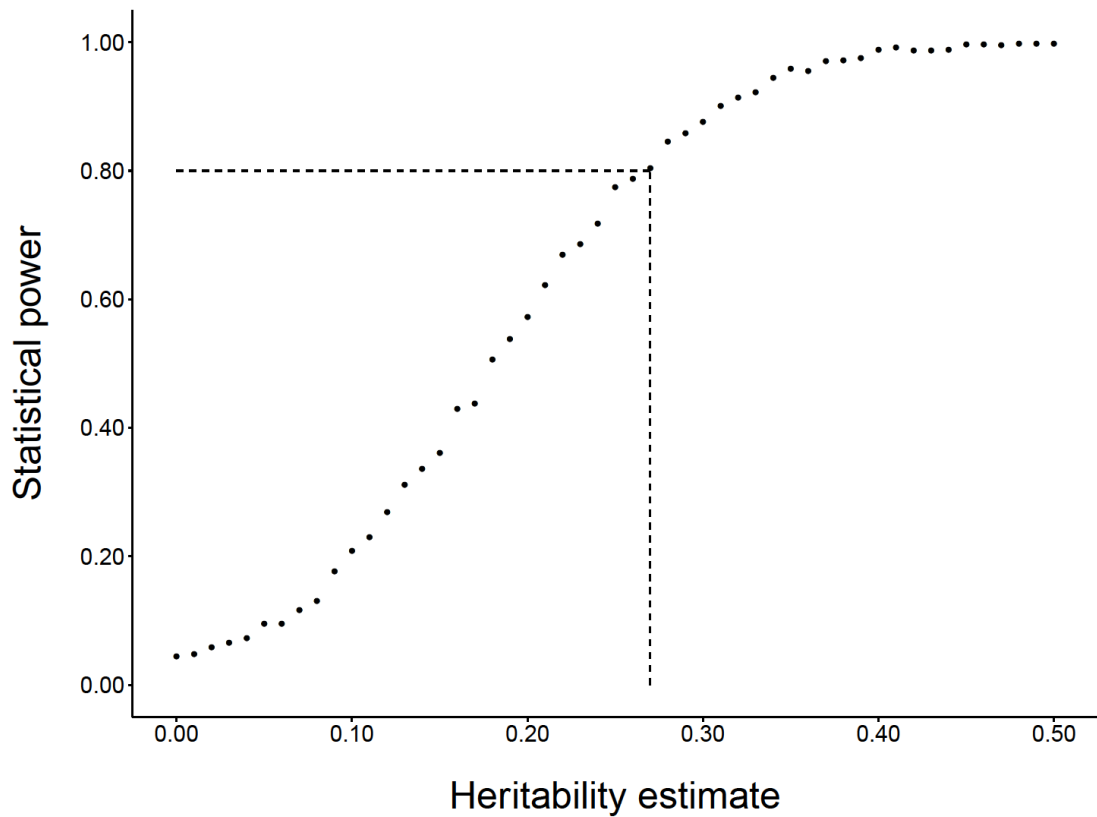
<sup>b</sup> Measurement error and not biological variance so no  $\chi^2$  and p-value provided



**Figure S5.1: Scatterplot showing the correlation between paternal and maternal ages at conception for badgers with relative leukocyte telomere length measures at any age ( $n = 471$  samples; 240 badgers). Parental ages are integers, jittered for clarity on the amount of data.**



**Figure S5.2: Statistical power to detect paternal age at conception (PAC) effect sizes in our European badger dataset using *simr* v1.0.5 (Green and MacLeod, 2016). Point estimates and error bars show mean power with associated 95% confidence intervals estimated from 500 simulations. Dashed line represents 80% power to detect a PAC effect size of 0.00067 or greater, with the specifications of our model and structure of our data. This is similar to a correlation coefficient of 0.131 (where correlation coefficient =  $(\beta_{PAC} * SD_{PAC}) / SD_{RTL} = (0.00067 * 24.57207) / 0.1254953$ ).**



**Figure S5.3: Statistical power to detect varying heritability estimates of telomere length in the European badger with our dataset ( $n = 1248$  measurements; 612 badgers) and pruned pedigree structure using *pedantics* 1.7 (Morrissey and Wilson, 2010). Point estimates show mean power estimated from 1000 simulations. Dashed line represents 80% power to detect a heritability estimate of 0.27 or greater, with the specifications of our model and structure of our data.**

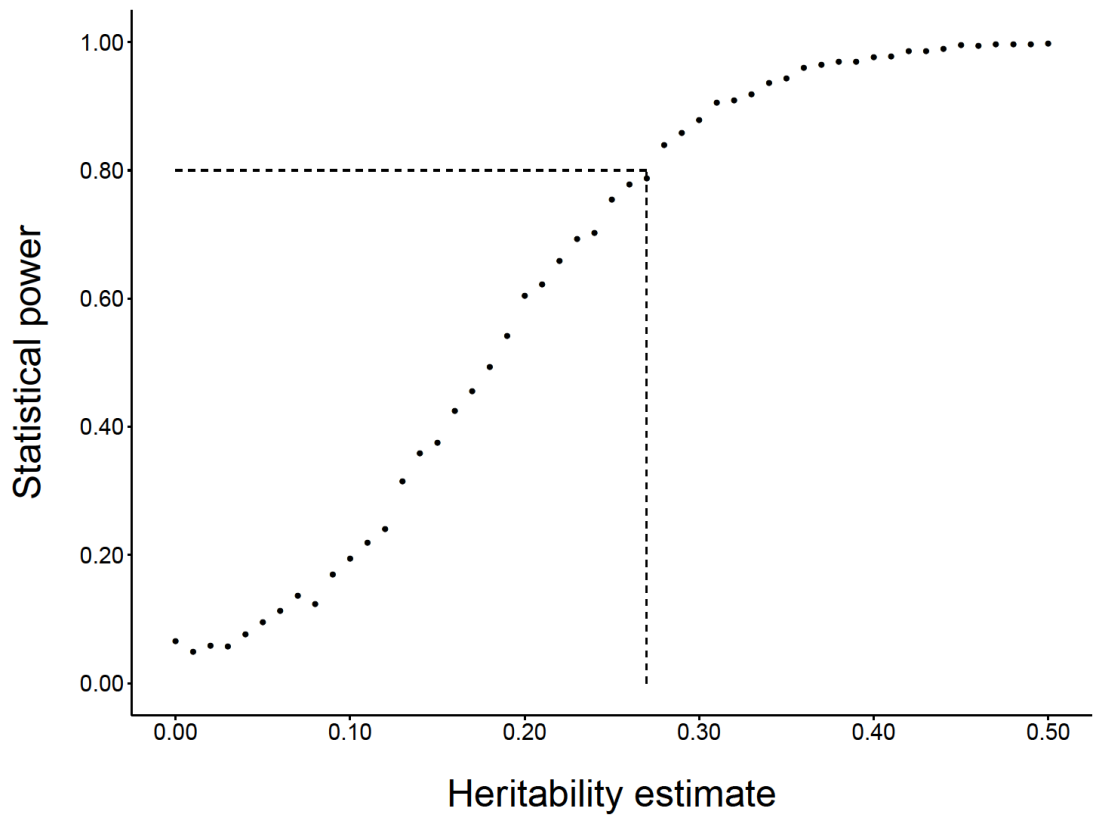


Figure S5.4: Statistical power to detect varying heritability estimates of juvenile telomere length ( $\leq 29$  months old) in the European badger with our dataset ( $n = 837$  measurements; 556 badgers) and pruned pedigree structure using *pedantics* 1.7 (Morrissey and Wilson, 2010). Point estimates show mean power estimated from 1000 simulations. Dashed line represents 80% power to detect a heritability estimate of 0.28 or greater, with the specifications of our model and structure of our data.



**Chapter 6: Testing the Predictive Adaptive Response: Consequences of early-life adversity for adaptive life-history strategies in a wild mammal**

## 6.1 Abstract

Early-life adversity can have long-term consequences for development and fitness, which may trigger adaptive life-history strategies based on the predictive adaptive response (PAR) hypotheses. The silver spoon hypothesis states that individuals born in favourable early-life conditions consistently outperform others born in adverse early-life conditions, irrespective of the adult environment. This is also predicted by the internal PAR hypothesis, but is adaptive as individuals may benefit from advancing their life-history schedules. These hypotheses are mutually non-exclusive with the external PAR hypothesis, where individuals use early-life adversity as a cue in anticipating the adult environment, and adapt their phenotype accordingly. Here, using a wild population of European badgers (*Meles meles*), we test the silver spoon, internal PAR and external PAR using early-life telomere length, and mean daily rainfall and temperature to quantify environmental quality. Autocorrelation among years was low for rainfall and moderate for temperature, with a correlation between early- and later-life mean daily temperature only. Refuting the silver spoon and internal PAR, early-life conditions did not significantly affect lifespan. However, individuals that experienced matching early- and later-life conditions did not have longer lifespans in terms of early-life telomere length and rainfall, but did when early- and later-life temperature conditions matched. Additionally, individuals experiencing higher mean daily rainfall or temperature across adulthood had longer lifespans. These results do not support the silver spoon or internal PAR, but show some evidence for the external PAR hypothesis when there is a moderate (auto)correlation, and indicate that mean environmental quality in adulthood shapes individual life-history.

## 6.2 Introduction

The mean of and variability in early-life environmental conditions can impact development, and have long-term effects on the expression of individual phenotypes (Fawcett and

Frankenhuis, 2015; Bateson et al., 2004), with consequences for survival to adulthood (Gaillard et al., 2000), physiology (McMillen and Robinson, 2005) and individual life-history strategies (Lindstrom, 1999; Lummaa and Clutton-Brock, 2002; Metcalfe and Monaghan, 2001; Cooper and Kruuk, 2018). Exposure to favourable early-life conditions can lead individuals to consistently outperform others experiencing adverse early-life conditions, regardless of the adult environment, termed 'the silver spoon hypothesis' (Grafen, 1988; Monaghan, 2008). Numerous studies have provided empirical evidence in support of the silver spoon hypothesis (van de Pol et al., 2006; Douhard et al., 2014; Nussey et al., 2007a; Reed et al., 2008; Millon et al., 2011), including a meta-analysis showing slower rates of reproductive senescence in favourable developmental environments (Cooper and Kruuk, 2018). However, in addition to the direct advantages implicit in silver spoon effects, individuals can also seek to mitigate the developmental and environmental challenges they face, through adopting adaptive life-history strategies.

Developmental plasticity in response to the environment can involve modifications to behaviour and physiology (Bateson et al., 2004; Nettle et al., 2013a; Dantzer et al., 2013; Auer, 2010), with consequences for the trade-off between survival and reproduction (Kirkwood and Rose, 1991; Stearns, 1992). For example, humans experiencing early-life adversity (e.g. low birth weight, psychosocial stress) have earlier ages at first reproduction (Nettle et al., 2011; Sloboda et al., 2007; Cooper et al., 1996), with additional experimental evidence from non-human mammals that early-life adversity results in individuals advancing their reproductive schedules (Maestripieri, 2005; Sloboda et al., 2009; Cameron et al., 2008a; Cameron et al., 2008b). Such changes in the trade-off between somatic and reproductive investment must have an adaptive basis, where two classes of adaptive responses can explain the link between early-life adversity and accelerated reproduction, both based on predictive adaptive responses (PARs).

The internal PAR hypothesis posits that where early-life adversity has lasting negative effects on an individual's somatic state, reducing longevity, it should be advantageous to advance the reproductive schedule accordingly (Nettle et al., 2013a; Wells, 2012), even at the expense of somatic development or maintenance (Kirkwood and Rose, 1991; Kirkwood, 2005). Adverse effects of early-life conditions on adult survival probability have been documented (Monaghan, 2008; Lummaa and Clutton-Brock, 2002; Lindstrom, 1999; Reid et al., 2006; Taborsky, 2006; Rickard et al., 2010), making it adaptive to advance reproduction. There is also direct evidence for the internal PAR which links, for example, poor quality early-life environments measured through glucocorticoids in Assamese macaques (*Macaca assamensis*) and winter resource availability in Svalbard reindeer (*Rangifer tarandus platyrhynchus*) to adaptive individual life-history strategies in these long-lived species (Berghanel et al., 2016; Douhard et al., 2016). The internal PAR is thus an adaptive response in anticipation of an individual's own future state, and could be particularly relevant for long-lived species without the need for environmental autocorrelation (Nettle et al., 2013a).

The internal PAR is mutually non-exclusive with the external PAR hypothesis, which posits that early-life conditions provide a basis against which the individual anticipates conditions into which it will mature, by developing the most appropriate phenotype (Gluckman et al., 2005a; Gluckman et al., 2005b). For example, early-life adversity can function as a cue for later-life adversity and be anticipated through accelerating reproductive schedules, providing higher fitness when early- and later-life conditions match. Indeed, mother red squirrels (*Tamiasciurus hudsonicus*) exposed to high density cues have offspring with increased growth rates, which adjusts the offspring phenotype to match the environment experienced at independence, enhancing offspring fitness (Dantzer et al., 2013). In zebra finches (*Taeniopygia guttata*), heat stress in early-life increases survival probability only when heat stress is again experienced in adulthood, whereas survival

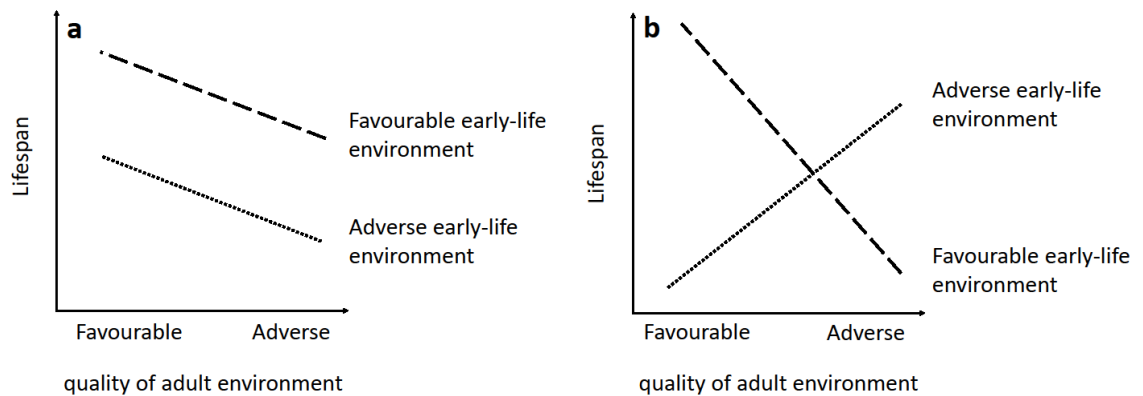
probability is reduced if heat stress is not encountered again (Costantini et al., 2014). A temporal mismatch between early and late-life conditions, limiting the ability to anticipate optimal life-history strategy effectively, can thus lead to fitness costs (Gluckman and Hanson, 2004; Gluckman et al., 2005a). In long-lived species, however, there is little evidence in support of the external PAR (Hayward and Lummaa, 2013; Hayward et al., 2013; Douhard et al., 2014; Lea et al., 2015; Pigeon et al., 2017). Furthermore, it has been questioned whether early-life conditions reliably predict future conditions (Wells, 2007), because year-to-year autocorrelation needs to be extremely high (>0.95 for human reproductive timing) for external PAR to be advantageous (Nettle et al., 2013a). Short-term fluctuations in environmental conditions may therefore reduce the predictive power of the future environment.

Quantification of the conditions experienced by an individual can be achieved through telomere length (Monaghan and Haussmann, 2006; Monaghan, 2010). Telomeres are hexameric tandem repeats (5'-TTAGGG-3') forming protective caps at the end of chromosomes that maintain genomic integrity (de Lange, 2004; Blackburn, 2000). Due to the end replication problem, telomeres shorten with age (Olovnikov, 1973), the rate of which is exacerbated by environmental stressors (Heidinger et al., 2012) and with *in vitro*, but no *in vivo*, evidence for oxidative damage (Reichert and Stier, 2017; von Zglinicki, 2002; Boonekamp, 2017). Somatic cells with critically short telomeres can enter replicative senescence, accumulate and impair tissue functioning due to reduced renewal capacity (Campisi, 2005; Campisi and di Fagagna, 2007). However, whether telomeres play a causal role in senescence, defined as declining performance with age, or act as a biomarker of somatic integrity in wild populations, remains unclear (Young, 2018). Telomere length has been linked to individual life-histories (Wilbourn et al., 2018), and often varies substantially between cohorts (Fairlie et al., 2016; Spurgin et al., 2017; van Lieshout et al., 2019). Adverse early-life conditions can therefore cause premature telomere shortening, affecting health

and somatic development, providing a tool to test the silver spoon, internal PAR and external PAR.

Here, we use data from a long-term study of European badgers (*Meles meles*; henceforth ‘badgers’) to test the silver spoon, internal and external PAR hypotheses. These badgers live in polygynandrous social groups (mean group size: 11.3, range: 2–29; da Silva et al., 1994; Macdonald et al., 2015). Mean litter size is  $1.4 \pm 0.06$  (range = 1–4; Dugdale et al., 2007), with cubs emerging from the communal underground den (termed setts) at 6–8 weeks old, being weaned at 12 weeks, and reaching independence at 14–16 weeks old (Fell et al., 2006; Dugdale et al., 2010). Cub survival probability ranges from 61–94% (mean  $\pm$  SE =  $0.67 \pm 0.03$ ; Macdonald et al., 2009), and cub cohorts are impacted by early-life exposure to gastro-intestinal coccidia infection (Newman et al., 2001), oxidative stress (Bilham et al., 2018) and weather conditions (Macdonald et al., 2010; Noonan et al., 2014; Nouvellet et al., 2013). Mean annual adult survival rate in this population is 0.83 ( $\pm 0.01$  SE; Macdonald et al., 2009) with a mean lifespan of 3.31 years ( $\pm 3.51$  SD; Bright Ross, J., Pers. Comm.).

We test for correlations between the early-life and adult environment experienced by an individual, to determine whether: (i) favourable early-life conditions result in a longer lifespan (i.e. silver spoon hypothesis and internal PAR; Figure 6.1a); (ii) matching early-life and later-life conditions result in longer lifespans (i.e. external PAR; Figure 6.1b); (iii) internal and external PAR co-occur since they are mutually non-exclusive and, (iv) autocorrelation in environmental quality among years occurs (required for the external PAR). We then discuss these findings in the context of the silver-spoon, internal PAR and external PAR hypotheses.



**Figure 6.1: Expected relationships between quality of the environment linked to lifespan for silver spoon and, internal and external predictive adaptive response (PAR) hypotheses. (a) silver spoon and internal PAR hypothesis, where individuals born in favourable conditions (dashed line) will always outperform individual born in adverse conditions (dotted line), irrespective of the quality of the adult environment. (b) external PAR hypothesis, individuals born in favourable conditions (dashed line) outperform individuals born in adverse conditions (dotted line) when the quality of the adult environment is favourable. In contrast, individuals born in adverse conditions may outperform individuals born in favourable conditions when the experiencing adverse adult environments.**

### 6.3 Methods

#### 6.3.1 Study population and trapping

Data for this study were collected in Wytham Woods, Oxfordshire, UK (51°46'24"N, 1°20'04"W), 6 km northwest of Oxford in southern England; a 424 ha mixed deciduous/coniferous secondary and ancient woodland surrounded by agricultural pasture (Macdonald and Newman, 2002; Macdonald et al., 2004b). The resident high-density badger population (range = 20.5–49.5 badgers/km<sup>2</sup>; Macdonald et al., 2015) is exposed to substantial inter-annual weather variation. The population comprises 19±2 (range = 14–26; Dugdale et al., 2008) mixed-sex social groups (Johnson et al., 2002; Newman et al., 2011) and is geographically discrete, which limits but not eliminates immigration and emigration (annually ca. <3%; Macdonald and Newman, 2002).

Badgers were captured three or four times per year since 1987, over two weeks in May, August and November, with 1 week of trapping in January in focal years. Captured badgers were anaesthetised using an intra-muscular injection of 0.2 ml ketamine

hydrochloride per kg body weight (McLaren et al., 2005) and given a unique tattoo number on the left inguinal region for permanent (re-)identification. Individuals were classified as cub (<1 year old) or adult ( $\geq 1$  year old), with further morphometric measurements (e.g. tooth wear; da Silva and Macdonald, 1989; Macdonald et al., 2009) and sett, social group and capture data recorded. The age of a badger (in months) was calculated as the difference between its date of birth (standardised on 14<sup>th</sup> February) and the capture date. Badgers first caught as adults were aged by tooth wear, following van Lieshout et al. (2019). Adults were assumed to have died in the last year they were caught. Jugular blood samples were collected via venipuncture into vacutainers with EDTA anticoagulant, and stored at -20°C immediately. After full recovery from anaesthesia, badgers were released at their setts of capture.

### **6.3.2 Telomere analyses**

DNA was extracted from whole blood samples ( $n = 332$  samples; 332 badgers), using the DNeasy Blood & Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's protocol, but with modifications using 125  $\mu$ l of anticoagulated blood and a double elution step (2x 75  $\mu$ l AE buffer). DNA integrity was checked by running a random selection of DNA extracts (ca. 20%) on agarose gels. Subsequently, DNA concentration was measured using the Fluostar Optima fluorometer (BMG Labtech, Ortenberg, Germany), standardized to 20 ng/ $\mu$ l and stored at -20 °C. Relative leukocyte telomere lengths were quantified using monochrome multiplex quantitative PCR (MMqPCR; Cawthon, 2009). This provides a ratio of copy numbers of telomeric sequence to a reference gene (T/S ratio), with both analysed in the same well to reduce measurement error. A detailed description of the MMqPCR analysis is given in van Lieshout et al. (2019).



### **6.3.3 Environmental quality implied through telomere length, and mean daily rainfall and temperature**

A previous study on Wytham badgers has shown that early-life telomere length functions as a biomarker of senescence (van Lieshout et al., 2019) and reflects the conditions experienced in early-life (Chapter 4). In badger adulthood, weather conditions (as a proxy for food availability) reflect environmental quality (Nouvellet et al., 2013; Noonan et al., 2014; Macdonald et al., 2010) with both mean daily rainfall and temperature having a beneficial effect on an individual's somatic state (i.e. early-life telomere length; Chapter 4). Thus, to characterise the later-life environment we first calculated the mean daily rainfall and temperature in each year of adulthood (Table S6.1) and then calculated the mean of the mean daily rainfall and temperature across adulthood as our measure of environmental quality.

### **6.3.4 Statistical analyses**

Statistical analyses were conducted in R 3.3.1 (R Development Core Team, 2019). Significance in generalised linear mixed models (GLMMs), using *lme4* 1.1-21 (Bates et al., 2015), was estimated with log-likelihood ratio tests, and set at  $P < 0.05$ . We also checked for collinearity (VIF < 3), and early-life RLTL was Z-transformed (Verhulst, 2019).

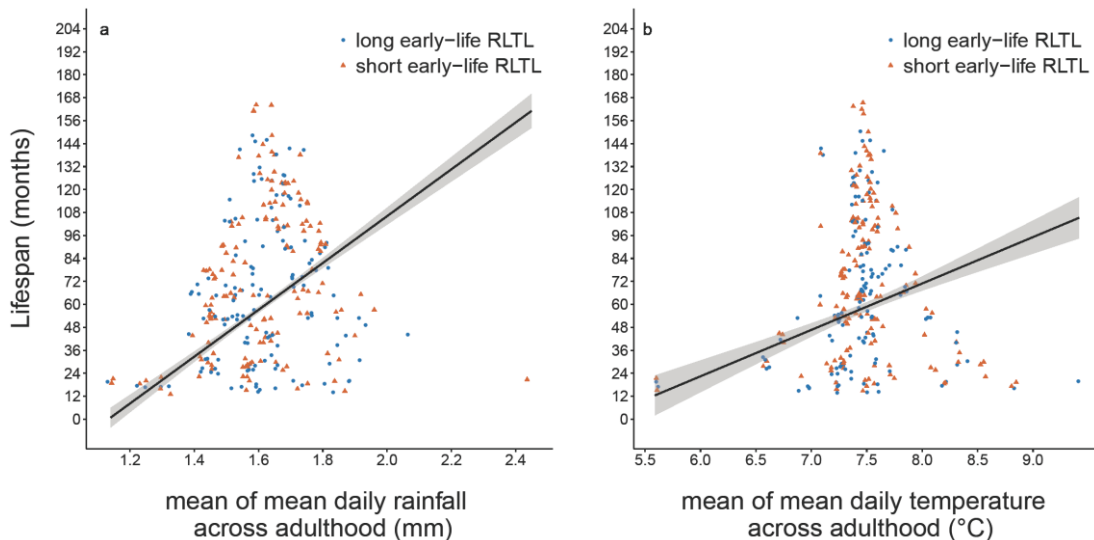
#### **(i) Silver spoon, internal PAR and external PAR**

We tested the relative importance of early-life, later-life and the similarity between these stages in a GLMM with lifespan as the response variable ( $n = 289$  observations;  $n = 289$  adult badgers) and a Poisson error distribution (log link function). Lifespan was the age at last capture. The model contained early-life RLTL (sex and age sampled do not affect early-life RLTL; van Lieshout et al., 2019), the mean of mean daily rainfall and temperature across adulthood, and the interaction between early-life RLTL and rainfall, and early-life RLTL and temperature as fixed effects. Cohort, social group, year and observation (for each unique

measure to account for overdispersion; Harrison, 2014) were included as random effects (Model 6.1). Secondly, we tested the association between early-life and later-life conditions on annual adult survival probability in a GLMM with adult survival probability from one year of adulthood to the next over the adult's lifespan as the response variable ( $n = 1529$  observations; 332 badgers) and a binomial error distribution (link = logit). Early-life RLTL, mean daily rainfall and temperature in each year of adulthood, and the interaction between early-life RLTL and rainfall, and early-life RLTL and temperature were included as fixed effects. Cohort, social group, year and individual ID were included as random effects (Model 6.2). Finally, we checked the robustness of our results by replacing early-life RLTL as a measure of early-life environmental quality by the mean daily rainfall and temperature in early-life, and its association with lifespan (Model 6.3) and adult survival probability (Model 6.4).

#### **(ii) Autocorrelation of early- and later-life environmental quality**

To determine the potential for external PAR, we used Pearson's correlation coefficients to first establish the correlation between early-life RLTL (<1 year old) and mean of mean daily rainfall or temperature across adulthood ( $\geq 1$  year old). Secondly, we extracted the correlation between early-life RLTL and mean daily rainfall or temperature in each year across an adult's lifespan, as the regression coefficient, from a GLMM with mean daily rainfall or temperature as the response variable, early-life RLTL as a fixed effect, and individual ID as a random effect. We also determined year-to-year autocorrelation ( $r$ ) for mean daily rainfall and temperature among years as a measure of consistency in environmental quality.



**Figure 6.2: The association between the environmental quality (a: mean in mean daily rainfall; b: temperature) across adulthood and lifespan, with data points coloured based on early-life environmental quality (early-life relative leukocyte telomere length; early-life RLTL). Raw data points ( $n = 289$  observations; 289 badgers) are shown and are modelled as a continuous variable in the mixed model, but for visualisation are plotted for cubs with low-quality early-life environment ( $n = 152$  observations, 152 badgers; brown triangles) and high-quality early-life environment ( $n = 137$  observations, 137 badgers; blue circles). The fitted line represents the model prediction, with associated 95% confidence intervals as shaded areas.**

## 6.4 Results

### (i) Silver-spoon, internal PAR and external PAR

Individuals for which early-life conditions, measured through early-life RLTL, matched their mean adult environment did not have longer lifespans (Table S6.2; Model 6.1). However, individuals experiencing a higher mean environmental quality across adulthood (in terms of both rainfall and temperature) had longer lifespans, irrespective of their early-life conditions (Figure 6.2; Table S6.2). When early-life and adult conditions matched there was no effect on annual adult survival probability (Table S6.2; Model 6.2), with no evidence for any separate effect of early-life or adult environmental quality. These results do not provide support for the silver spoon, internal PAR or external PAR hypothesis or a combination of these. The results are robust when using mean daily rainfall in early-life instead of early-life RLTL (Table S6.2; Model 6.3 & 6.4). However, when using temperature, individuals had longer

lifespans when mean daily temperature in early-life and the mean of mean daily temperature across adulthood matched (Table S6.2; Figure 6.3; Model 6.3), but this effect was much weaker than the main effect of mean daily temperature across adulthood on lifespan (Table S6.2).

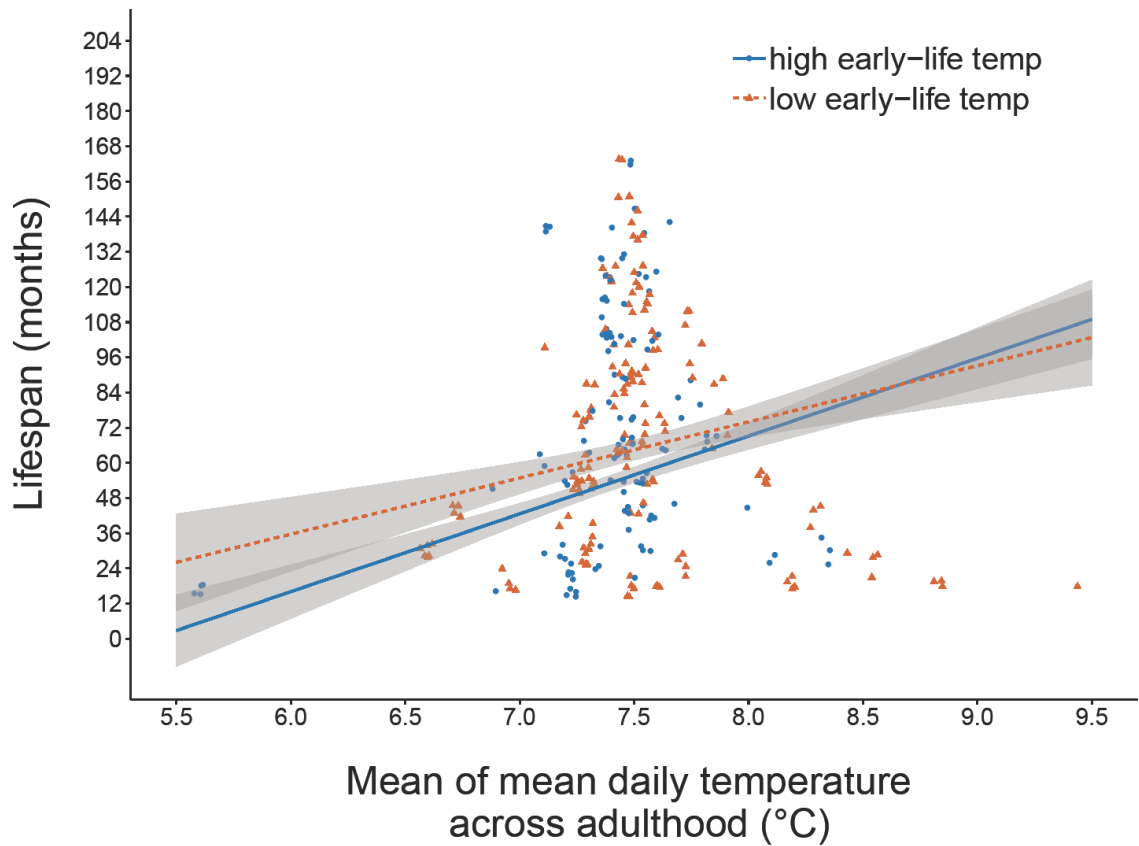
**Table 6.1: Correlation coefficients between early- and late-life metrics of environmental quality. RLTL = relative leukocyte telomere length.  $r$  = Pearson’s correlation coefficient, or extracted from a generalised linear mixed model through the regression coefficient. Significant correlations ( $P < 0.05$ ) are in bold.**

	Lifespan ( $n = 289$ )		Annual survival probability ( $n = 1529$ )	
	Mean of mean daily rainfall across adulthood	Mean of mean daily temperature across adulthood	Mean daily rainfall in adulthood	Mean daily temperature in adulthood
Early-life RLTL	$r = 0.048$ ; $p = 0.418$	$r = 0.031$ ; $p = 0.597$	$r = -0.005$ ; $p = 0.550$	$r = -0.001$ ; $p = 0.972$
Mean daily rainfall in early-life	$r = -0.065$ ; $p = 0.272$		$r = -0.001$ ; $p = 0.910$	
Mean daily temperature in early-life		<b><math>r = -0.202</math>;</b> <b><math>p &lt; 0.001</math></b>		<b><math>r = -0.051</math>;</b> <b><math>p = 0.024</math></b>

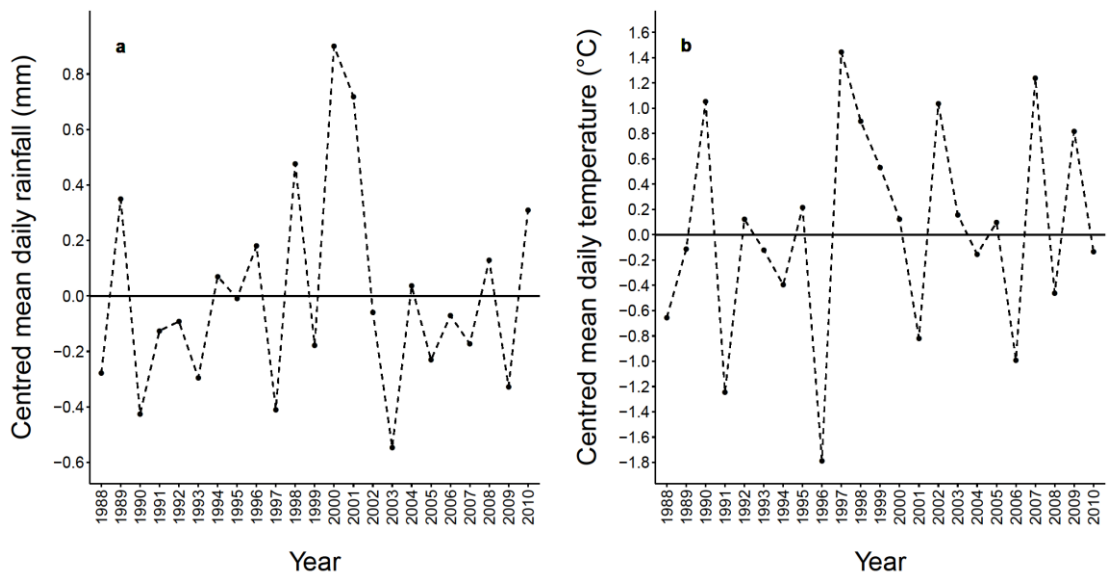
**(ii) Autocorrelation and early- and later-life environment quality**

Autocorrelation in mean daily rainfall among years was low ( $r = -0.076$ ,  $n = 23$ ; Figure 6.4a), whereas for mean daily temperature there was a moderate year-to-year autocorrelation ( $r = -0.400$ ,  $n = 23$ ; Figure 6.4b), indicating little to moderate consistency in consecutive inter-annual environmental conditions. We found no significant correlation between early-life RLTL and the mean of mean daily rainfall or temperature across adulthood, or the mean daily rainfall or temperature in each year of adulthood (Table 6.1; Figure S6.1 & S6.2). Even though mean daily rainfall in early-life was not significantly correlated with the mean of mean daily rainfall across adulthood or in each year of adulthood, there were correlations for mean daily

temperature in early-life with both the mean of mean daily temperature across adulthood and the mean temperature in each year of adulthood (Table 6.1; Figure S6.1 & S6.2).



**Figure 6.3:** The interaction between mean daily temperature in the natal year and mean of mean daily temperature across adulthood on lifespan. Raw data points ( $n = 289$ ) are jittered for clarity on the amount of data, and modelled as continuous variables, but for visualisation shown for individuals with low mean daily temperatures ( $n = 165$ ; brown, triangle, dashed) and high mean daily temperatures in the natal year ( $n = 124$ ; blue, circle, solid). Fitted lines represent the model prediction for mean daily temperature interacting with the mean of mean daily temperature across adulthood, with associated 95% confidence intervals as shaded areas.



**Figure 6.4: Centred mean daily rainfall (a) and temperature (b) over February to May from 1987 to 2010. The mean in mean daily rainfall and temperature from 1987 to 2010 was subtracted from the mean daily rainfall and temperature, respectively, in each year to generate a centred mean value.**

## 6.5 Discussion

We found no evidence in support of the external PAR in badgers using early-life RLTL, since individuals that experienced matching early- and later-life conditions did not have longer lifespans. However, we did find some evidence for the external PAR using temperature as a measure of early- and later-life environmental quality, but not when using rainfall. Most support for the external PAR hypothesis comes short-lived species (Lee and Zucker, 1988; van den Heuvel et al., 2013; Dantzer et al., 2013), but the applicability of such effects in long-lived species have been questioned (Wells, 2006; Wells, 2007; Kuzawa, 2005). Studies in long-lived species have tested for but did not find supportive evidence for the external PAR (Douhard et al., 2014; Lea et al., 2015; Hayward et al., 2013; Pigeon et al., 2017). The discrepancy between short- and long-lived species is likely due to faster maturation in short-lived species and therefore a stronger correlation between the early- and late-life environment (Wells, 2006). The relatively slow maturation of badgers, compared to short-lived species, and high fluctuation in environmental conditions in this population makes matching early- and later-life conditions less likely.

Autocorrelation in the year-to-year environment was low for rainfall ( $r = -0.076$ ) and moderate for temperature ( $r = -0.400$ ), indicating little to moderate consistency in environmental quality among years. For an external PAR in reproductive timing in humans the autocorrelation needs to be extremely high ( $>0.95$ ; Nettle *et al.* 2013). This suggests that great environmental persistence is required for predictive plasticity to be beneficial (Fischer *et al.*, 2011; Reed *et al.*, 2010; Padilla and Adolph, 1996) and that temporal fluctuations in the environment reduce the benefits of the external PAR as an adaptive strategy (Wells, 2007; Rickard and Lummaa, 2007). Similarly, we found no evidence for an external PAR relationship using rainfall as a measure of environmental quality, where there was low autocorrelation between years. We did however find moderate autocorrelation in temperature between years and a correlation between early- and later-life mean daily temperatures, with support for the external PAR even though the effect size was small. Since badgers are dependent on weather conditions such as rainfall and temperature for food availability, which do not show high levels of autocorrelation, the potential for external PAR based on environmental autocorrelation is small.

While we show weak or no evidence for the external PAR, depending on the metric of environmental quality and the strength of its autocorrelation, we found a much stronger effect of the mean environmental quality across adulthood on individual lifespan, but no effect on annual adult survival probability. With the relatively low consistency in environmental quality, life-history decisions (i.e. whether to invest in reproduction) depend on the cumulative conditions, not just over a single year, experienced as an adult. However, a large proportion of the variance in lifespan was dependent on cohort (45%), compared to year (5.5%), which is likely due to individual lifespan being dependent on survival to adulthood when mortality in badgers is highest (Macdonald and Newman, 2002). In contrast, for annual adult survival probability, year (29%) explained much more of the variance than cohort ( $<0.01\%$ ) and is thus more dependent on the environmental quality in adulthood.

Thus, while we found evidence for the external PAR, the stronger effect of the mean adult environment on lifespan indicates stage-dependent effects with early-life environment defining survival to adulthood and mean adult environmental quality shaping life-histories.

We found no support for the silver spoon or internal PAR hypotheses, as neither longevity nor annual adult survival probability depended on the quality of the early-life environment. Badger cubs are exposed to social conditions and weather variability, which can have detrimental consequences on an individual's somatic state (Chapter 4). Such early-life adversity can thus detrimentally affect the soma and it may be beneficial to have adaptive life-history strategies based on developmental history (Nettle et al., 2013a). While support for such detrimental effects of early-life conditions on life-history is abundant (e.g. Lindstrom, 1999; Lummaa and Clutton-Brock, 2002; Monaghan, 2008), we found no long-term effects of early-life adversity and do not detect evidence for the internal PAR even though other studies in long-lived species have (Berghanel et al., 2016; Douhard et al., 2016).

In conclusion, we provide no evidence in support of the silver spoon or internal PAR hypothesis, but show weak evidence for the external PAR in a long-lived species. The effects of early-life conditions leading to constrained development of the individual's physiology and soma do not lead to adaptive life-history strategies in badgers. Even though we found that matching early-life and later-life conditions can have beneficial effects for fitness in response to the anticipated adult environment, when autocorrelation is at least moderate ( $>0.40$ ), individual life-histories in badgers are defined by the strong effect of mean adult environmental quality. Particularly in long-lived species the lack of cue validity of the adult environment and current increases in environmental variability due to climate change (IPCC, 2014) may incapacitate adaptive plastic responses to environmental change. The adaptive value of adjusting life-history strategies in response to early- and later-life conditions, and the effects on the soma, requires further understanding to link life-history evolution and the ability of wild populations to cope with changing conditions.



## Supplemental information Chapter 6: Testing the Predictive Adaptive Response: Consequences of early-life adversity for adaptive life-history strategies in a wild mammal

**Table S6.1: Mean daily rainfall (mm;  $\mu_{rain}$ ) and mean daily temperature ( $^{\circ}\text{C}$ ;  $\mu_{temp}$ ) estimates with standard deviation (SD) for the years 1987 – 2010.**

Year	$\mu_{rain}$ ( $\pm$ SD)	$\mu_{temp}$ ( $\pm$ SD)
1987	1.597 ( $\pm$ 2.805)	6.509 ( $\pm$ 4.253)
1988	1.270 ( $\pm$ 2.422)	6.722 ( $\pm$ 2.833)
1989	1.898 ( $\pm$ 3.225)	7.263 ( $\pm$ 2.463)
1990	1.123 ( $\pm$ 2.739)	8.429 ( $\pm$ 2.994)
1991	1.424 ( $\pm$ 3.531)	6.131 ( $\pm$ 4.222)
1992	1.457 ( $\pm$ 2.845)	7.499 ( $\pm$ 2.704)
1993	1.254 ( $\pm$ 3.058)	7.256 ( $\pm$ 3.386)
1994	1.619 ( $\pm$ 2.743)	6.981 ( $\pm$ 3.567)
1995	1.540 ( $\pm$ 2.584)	7.594 ( $\pm$ 2.938)
1996	1.729 ( $\pm$ 3.403)	5.590 ( $\pm$ 3.505)
1997	1.140 ( $\pm$ 2.995)	8.821 ( $\pm$ 2.272)
1998	2.025 ( $\pm$ 3.414)	8.273 ( $\pm$ 2.564)
1999	1.371 ( $\pm$ 2.191)	7.910 ( $\pm$ 3.521)
2000	2.449 ( $\pm$ 5.504)	7.501 ( $\pm$ 2.468)
2001	2.268 ( $\pm$ 4.009)	6.557 ( $\pm$ 3.138)
2002	1.491 ( $\pm$ 2.844)	8.415 ( $\pm$ 2.649)
2003	1.002 ( $\pm$ 2.583)	7.536 ( $\pm$ 3.983)
2004	1.585 ( $\pm$ 2.692)	7.223 ( $\pm$ 3.668)
2005	1.318 ( $\pm$ 2.997)	7.475 ( $\pm$ 4.132)
2006	1.478 ( $\pm$ 2.957)	6.383 ( $\pm$ 3.780)
2007	1.376 ( $\pm$ 2.596)	8.616 ( $\pm$ 3.342)
2008	1.678 ( $\pm$ 5.026)	6.914 ( $\pm$ 2.776)
2009	1.221 ( $\pm$ 3.467)	8.196 ( $\pm$ 3.064)
2010	1.857 ( $\pm$ 3.541)	7.244 ( $\pm$ 3.584)

**Table S6.2: Parameter estimates and 95% confidence intervals of fixed effects from mixed model testing early-life and later-life conditions, and their similarity, on lifespan in European badgers.  $\beta$  = direction and magnitude of effect, S.E. = standard error, 95% CI = 95% confidence interval,  $\chi^2$  = chi-squared value; reference terms in brackets = reference level for factors; \* = interaction. RLTL = relative leukocyte telomere length (Z-transformed). Significant parameters ( $p < 0.05$ ) are in bold.**

<b>Parameter (reference level)</b>	<b><math>\beta</math></b>	<b>S.E.</b>	<b>95% CI</b>	<b><math>\chi^2</math></b>	<b><math>p</math>-value</b>
<b>Model 6.1, Response: Lifespan</b>					
Intercept	4.005	0.138	3.719 to 4.289		
Early-life RLTL	-0.007	0.030	-0.066 to 0.053	0.047	0.828
<b>Mean of mean daily rainfall across adulthood</b>	<b>0.325</b>	<b>0.040</b>	<b>0.246 to 0.404</b>	<b>57.28</b>	<b>&lt;0.001</b>
<b>Mean of mean daily temperature across adulthood</b>	<b>0.175</b>	<b>0.036</b>	<b>0.104 to 0.245</b>	<b>22.63</b>	<b>&lt;0.001</b>
Early-life RLTL * Mean of mean daily rainfall across adulthood	0.007	0.032	-0.055 to 0.070	0.054	0.816
Early-life RLTL * Mean of mean daily temperature across adulthood	-0.002	0.031	-0.063 to 0.060	0.002	0.961
<b>Model 6.2, Response: Annual adult survival probability</b>					
Intercept	1.499	0.141	1.219 to 1.807		
Early-life RLTL	0.044	0.074	-0.100 to 0.194	0.347	0.556
Mean daily rainfall in adulthood	-0.055	0.103	-0.284 to 0.154	0.283	0.595
Mean daily temperature in adulthood	-0.162	0.110	-0.402 to 0.065	2.066	0.151
Early-life RLTL * Mean daily rainfall in adulthood	0.006	0.076	-0.144 to 0.158	0.006	0.939
Early-life RLTL * Mean daily temperature in adulthood	0.027	0.078	-0.128 to 0.181	0.114	0.736
<b>Model 6.3, Response: Lifespan</b>					
Intercept	4.056	0.130	3.788 to 4.323		
Mean daily rainfall in natal year	-0.027	0.109	-0.255 to 0.198	0.062	0.804
<b>Mean daily rainfall in adulthood</b>	<b>0.289</b>	<b>0.042</b>	<b>0.206 to 0.372</b>	<b>42.99</b>	<b>&lt;0.001</b>
Mean daily temperature in natal year	-0.137	0.103	-0.349 to 0.079	1.655	0.198
<b>Mean daily temperature in adulthood</b>	<b>0.167</b>	<b>0.036</b>	<b>0.096 to 0.239</b>	<b>20.13</b>	<b>&lt;0.001</b>
Mean daily rainfall in natal year * Mean daily rainfall in adulthood	0.060	0.059	-0.056 to 0.177	1.036	0.309
<b>Mean daily temperature in natal year * Mean daily temperature in adulthood</b>	<b>0.077</b>	<b>0.036</b>	<b>0.006 to 0.148</b>	<b>4.576</b>	<b>0.032</b>
<b>Model 6.4, Response: Annual adult survival probability</b>					
Intercept	1.495	0.137	1.222 to 1.790		
Mean daily rainfall in natal year	0.028	0.076	-0.121 to 0.182	0.137	0.712
Mean daily rainfall in adulthood	-0.063	0.100	-0.279 to 0.137	0.403	0.525

Mean daily temperature in natal year	-0.031	0.077	-0.183 to 0.122	0.159	0.690
Mean daily temperature in adulthood	-0.163	0.104	-0.391 to 0.053	2.312	0.128
Mean daily rainfall in natal year	0.076	0.074	-0.068 to 0.225	1.055	0.304
* Mean daily rainfall in adulthood					
Mean daily temperature in natal year * Mean daily temperature in adulthood	0.001	0.080	-0.156 to 0.160	0.001	0.988

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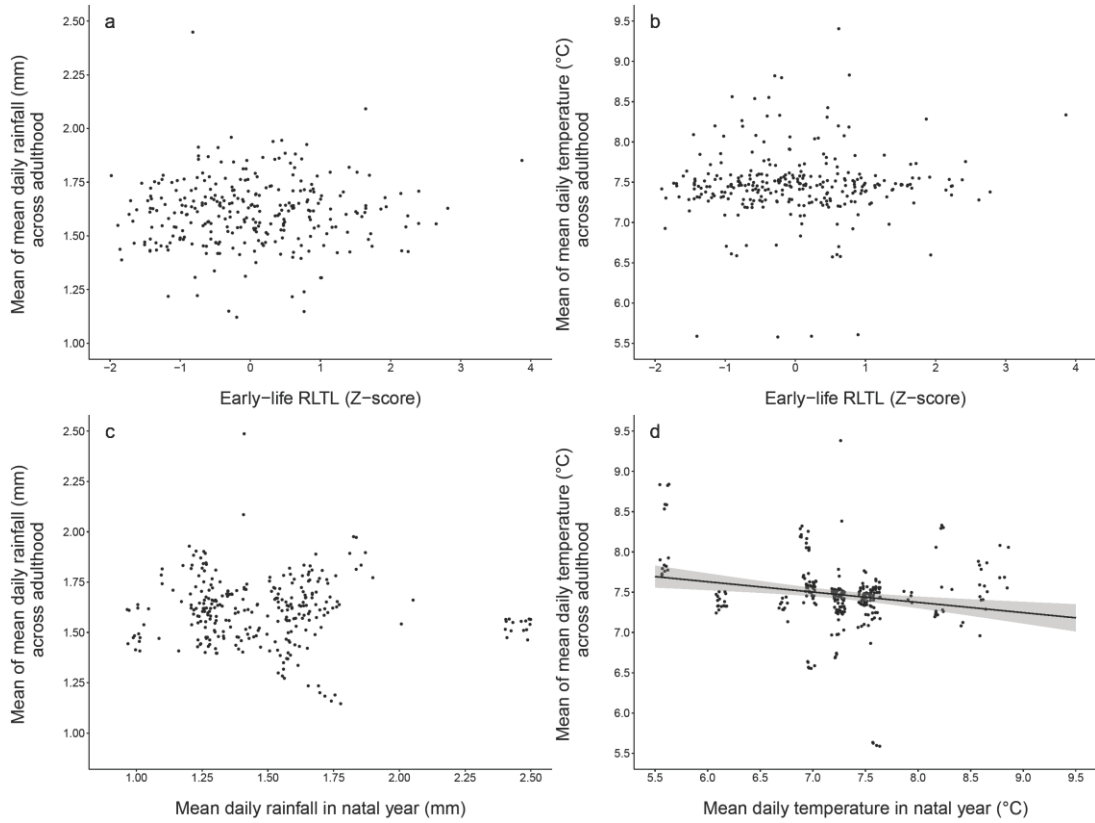
Random effect estimates (variance):

Model 1: Social group ( $3.343 \times 10^{-2}$ ), Cohort (0.2817), Year ( $2.828 \times 10^{-2}$ ), Observation (0.1677)

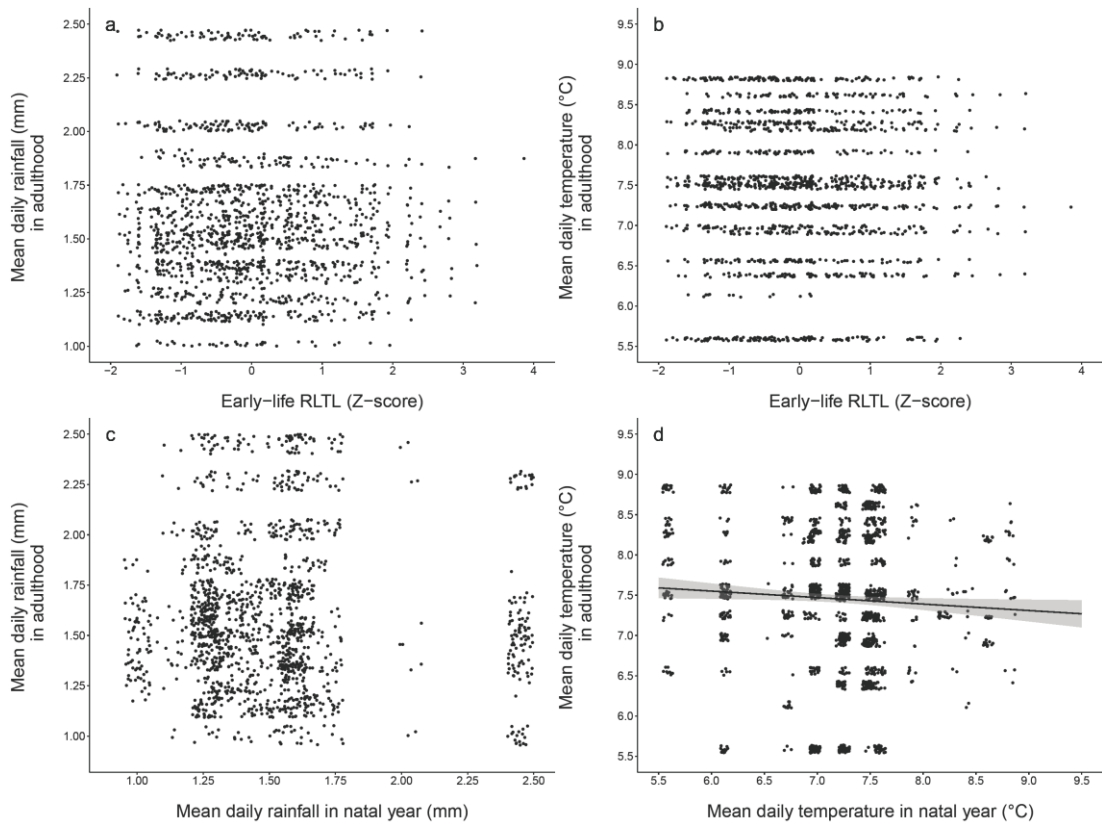
Model 2: Social group (0.2167), Cohort ( $<1.000 \times 10^{-12}$ ), Individual ID ( $<1.000 \times 10^{-12}$ ), Year ( $8.774 \times 10^{-2}$ )

Model 3: Social group ( $3.806 \times 10^{-2}$ ), Cohort (0.2247), Year ( $2.417 \times 10^{-2}$ ), Observation (0.1646)

Model 4: Social group (0.2100), Cohort ( $<1.000 \times 10^{-12}$ ), Individual ID ( $<1.000 \times 10^{-12}$ ), Year ( $7.130 \times 10^{-2}$ )



**Figure S6.1: Scatterplots showing the relationships between early-life relative leukocyte telomere length (RLTL) and the mean of mean daily rainfall (a) or temperature (b) across adulthood, or between mean daily rainfall (c) or temperature (d) and the associated means across adulthood ( $n = 289$  observations; 289 badgers). Data points are jittered for clarity on the amount of data, with significant regression lines plotted.**



**Figure S6.2:** Scatterplots showing the relationships between early-life relative leukocyte telomere length (RLTL) and mean daily rainfall (a) or temperature (b) in each year of adulthood, or between mean daily rainfall (c) or temperature (d) and the associated means in each year of adulthood ( $n = 1529$  observations; 332 badgers). Data points are jittered for clarity on the amount of data, with significant regression lines plotted.

## **Chapter 7: Slicing: a sustainable approach to structuring samples for analysis in long-term studies**

*The following paper, van Lieshout S.H.J., Froy, H., Schroeder, J., Burke, T., Simons, M.J.P., Dugdale, H.L. (2020) Slicing: a sustainable approach to structuring samples for analysis in long-term studies. Methods in Ecology and Evolution (in press), is a modified version of this chapter*

I was responsible for conception and development of the study and I co-wrote the simulations, conducted statistical analyses and manuscript writing. Co-authors developed the ideas and provided comments on draft manuscripts.

## **Glossary**

**Batch** – a set of analysed samples that are inherently dependent on one another, e.g. all using the same standard curve, machine, time of day, technician or that are equally affected by any other source of variation.

**Cluster** – a set of samples that are distinct in the timing of their analysis; this typically includes multiple batches nested within clusters.

**Nested** – all units at a lower hierarchical level are within one unit at a higher hierarchical level

**Cross-classified** – units at a lower hierarchical are associated with more than one unit at a higher hierarchical level.

**Within-individual effects** – Longitudinal changes within individuals in a repeatedly measured trait.

**Between-individual effects** – Comparison of differences in mean traits among individuals.

**Statistical power** – The ability to reject the null hypothesis when false, quantified by the proportion of significant values ( $P < 0.05$ ) out of the total.

**Precision** – The degree to which simulations provide similar results, quantified by the absolute difference between the 75% and 25% percentile divided by the median.

**Slicing** – Recently added samples analysed in clusters together with previously obtained samples, ensuring statistical independence of collection time and cluster.

## **7.1 Abstract**

1. The longitudinal study of populations is a core tool for understanding ecological and evolutionary processes. Long-term studies typically collect samples repeatedly over individual lifetimes and across generations. These samples are then analysed in batches (e.g. qPCR-plates) and clusters (i.e. group of batches) over time in the laboratory. However, these analyses are constrained by cross-classified data structures introduced

biologically or through experimental design. The separation of biological variation from the confounding among-batch and among-cluster variation is crucial, yet often ignored.

2. The commonly used approaches to structuring samples for analysis, sequential and randomisation, generate bias due to the non-independence between time of collection and the batch and cluster they are analysed in. We propose a new sample structuring strategy, called slicing, designed to separate confounding among-batch and among-cluster variation from biological variation. Through simulations we tested the statistical power and precision to detect within-individual, between-individual, year and cohort effects of this novel approach.
3. Our slicing approach, whereby recently and previously collected samples are sequentially analysed in clusters together, enables the statistical separation of collection time and cluster effects by bridging clusters together, for which we provide a case study. Our simulations show, with reasonable slicing width and angle, similar precision and similar or greater statistical power to detect year, cohort, within- and between-individual effects when samples are sliced across batches, compared with strategies that aggregate longitudinal samples or use randomised allocation.
4. While the best approach to analysing long-term datasets depends on the structure of the data and questions of interest, it is vital to account for confounding among-cluster and batch variation. Our slicing approach is simple to apply and creates the necessary statistical independence of batch and cluster from environmental or biological variables of interest. Crucially, it allows sequential analysis of samples and flexible inclusion of current data in later analyses without completely confounding the analysis. Our approach maximises the scientific value of every sample, as each will optimally contribute to unbiased statistical inference from the data. Slicing thereby maximises the power of growing biobanks to address important ecological, epidemiological and evolutionary questions.

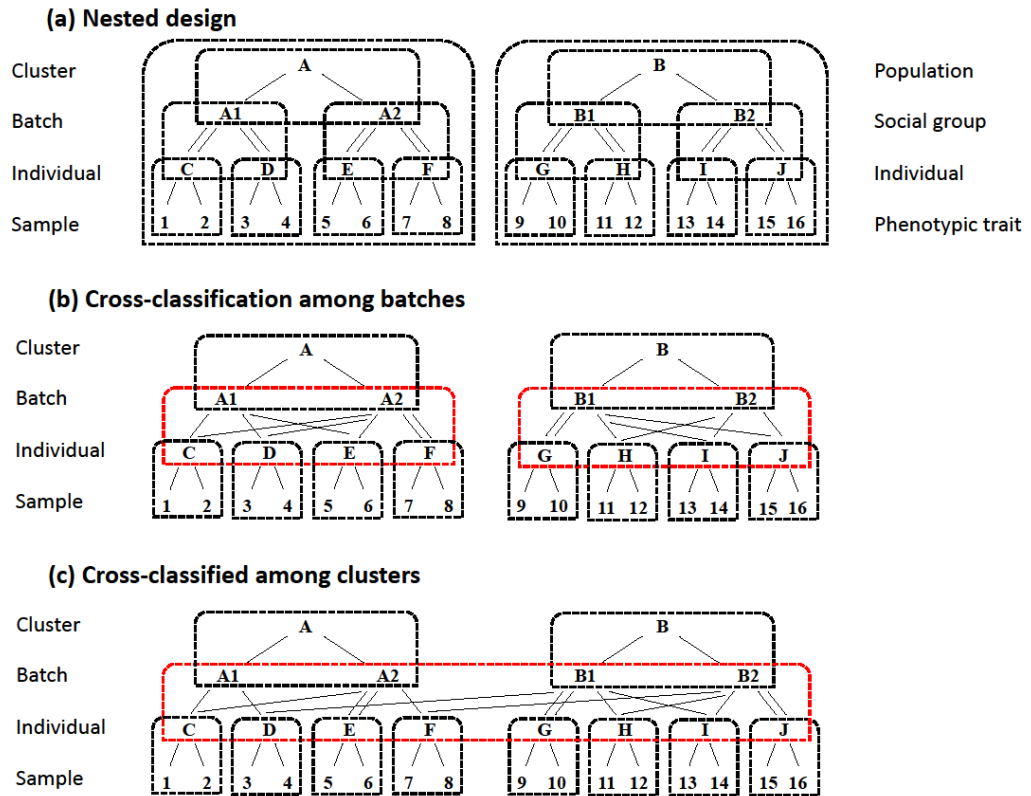


## 7.2 Introduction

Individuals and populations are shaped by ecological and evolutionary processes which generally occur over many years or decades (Clutton-Brock and Sheldon, 2010). Consequently, long-term studies are key in determining the proximate and ultimate causes of biological processes. Sampling a population repeatedly over individual lifetimes and across multiple generations allows quantification and separation of genetic variation from environmental variation and estimation of such effects with appropriate precision and statistical power (Martin et al., 2011; van de Pol, 2012). However, statistical analyses of such comprehensive biological datasets are often complex due to hierarchically structured data and difficulties in separating variation from sources of interest and confounding variables.

Due to the hierarchical nature of biology, for example, phenotypic traits nested within individuals, individuals nested within social groups and social groups nested within populations (Figure 7.1a), appropriate statistical methods are required that model the hierarchical structure of biological datasets. While nested designs, either natural or through experimental design (Figure 7.1a), can be analysed in linear models, this inflates the degrees of freedom and thus reduces statistical power (Gelman, 2005; Quinn and Keough, 2002; Underwood, 1997). A better approach is the mixed model framework, which estimates fixed effects while flexibly accounting for the variance explained by random effects, incorporating multilevel hierarchies in data (Bolker et al., 2009; Gelman and Hill, 2006; Snijders and Bosker, 2011; Zuur et al., 2010). However, in cross-classified designs, where one individual is associated with more than one batch (Figure 7.1b) or even more than one cluster (Figure 7.1c), advanced statistical methods to estimate fixed effects and variance components are required compared to nested designs (Schielzeth and Nakagawa, 2013). While cross-classified data structures in short-term studies are often the result of the experimental

design (e.g. cross-fostering), in long-term studies the timing of the analyses of data often naturally lead to cross-classification of data (Figure 7.1b–7.1c).



**Figure 7.1: Schematic of nested and cross-classified data structures: (a) With a nested design applied to populations and laboratory analyses; (b) Cross-classification of data among batches that is confounded by time of analysis; and (c) cross-classification common in longitudinal data in laboratory analyses across clusters. Black dashed delineation indicates nested, whereas red dashed delineation indicates cross-classified structures.**

In long-term studies, the individual-based collection of longitudinal data and biological samples from natural or laboratory populations produces large, continuously growing biobanks (Clutton-Brock and Sheldon, 2010). Through laboratory analyses these biobanks provide information on, for example, individual telomere length (Boonekamp et al., 2014; Fairlie et al., 2016), serological values (Andraud et al., 2014; Telfer et al., 2008) and genetic variation (Berry et al., 2012; Tollenaere et al., 2012). However, the laboratory analysis

of samples from growing biobanks is often conducted on separate groups of samples over time (e.g. after each fieldwork season, each year or coinciding with grant cycles). Such a group of samples – a cluster – will be collectively analysed under similar conditions, but these conditions might differ between clusters (e.g. different analyst, machine or month). Samples within a cluster are often further subdivided into batches (e.g. qPCR-plates) where, again, samples are analysed under similar conditions, but conditions may vary between batches (e.g. different reagents or day). While batches are nested within clusters, the continuous collection of samples in the field and intervals between laboratory analyses result in longitudinal samples from a single individual that may not be nested within batches or even clusters, causing cross-classified data structures in long-term studies (Gelman and Hill, 2006; Figure 7.1b–7.1c).

Cross-classification of data induces variation that can be confounded with the independent variables of interest, which can reduce the ability to compare results across samples and draw reliable conclusions (Greenland et al., 1999; Schielzeth and Nakagawa, 2013). This is problematic if cross-classification is not explicitly accounted for, or there is not sufficient cross-classification to disentangle these sources of variation with high statistical power. For example, temporal variation or, where multiple populations are studied, spatial differences in resource availability can be confounded with laboratory analysis when samples are analysed after each period of collection, resulting in a failure to separate the effects of resource availability and laboratory analysis on a response variable. The experimental design and therefore the method in structuring samples for clusters and allocating samples to batches requires consideration to cope with cross-classified data structures and confounding variables.

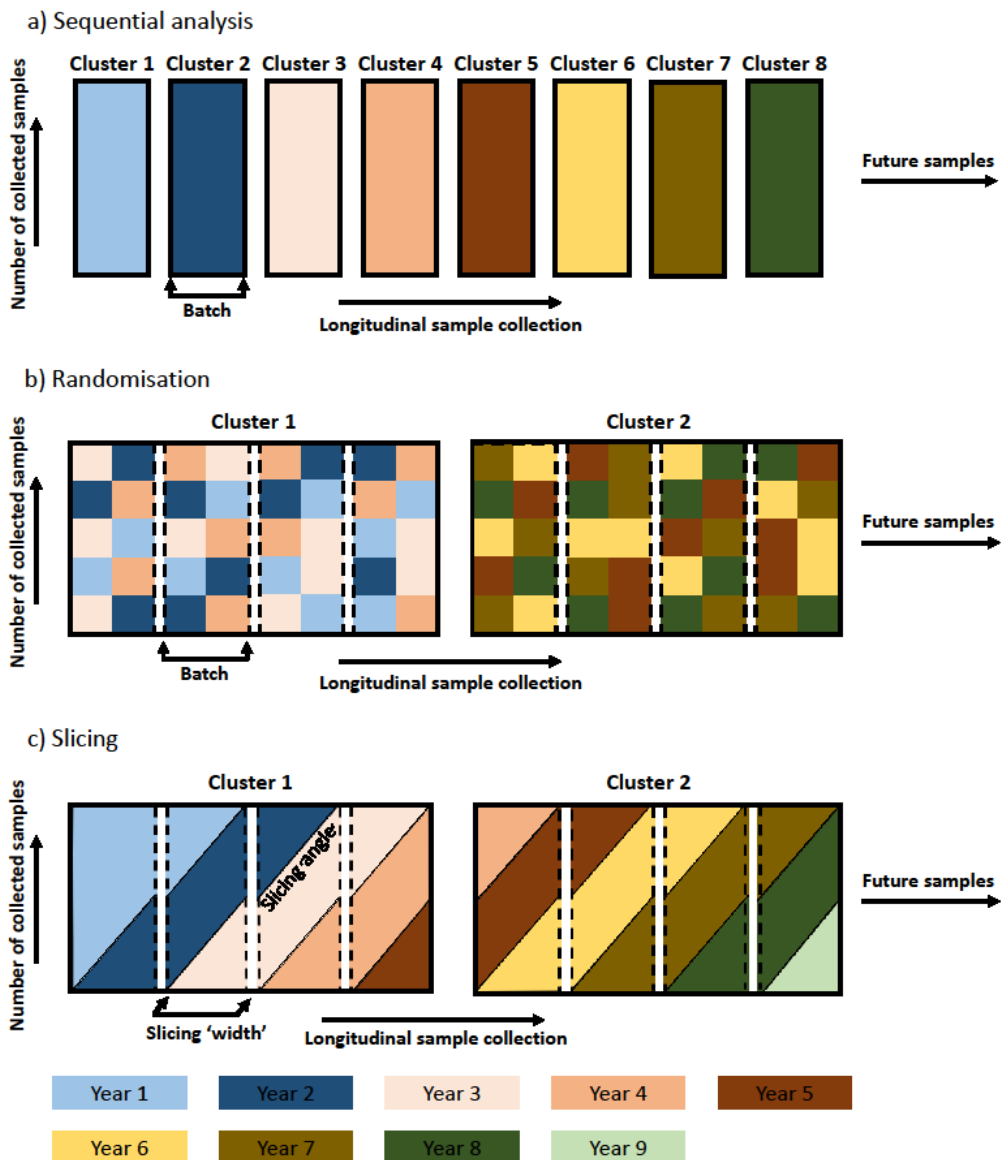


Figure 7.2: Schematic of three strategies to structure samples from the biobank. The sequential analysis strategy (a) can confound cluster and year, while randomisation of multiple years within a cluster (b) prevents this confound but generates uncontrollable variation between clusters. The slicing approach (c) combines the advantages of these approaches and can be used to sequentially analyse growing biobanks, while maintaining independence between cluster and associated variables. The biobank is sliced (e.g. by year), thereby analysing a set of continuously collected samples sequentially in each subsequent cluster. Each sample only needs to be analysed once, where different samples from the same slice are analysed across batches and clusters (e.g. year 4 and 5), which enables controlling for batch and cluster effects. Slicing width (frequency of new samples collected) and angle (degree of independence between slices) determine the level of statistical independence between clusters.

While relatively few studies report the approach used to structure samples into clusters, currently two main approaches are used, and both are prone to confounding effects and cross-classified data structures. First, sequential structuring of samples to clusters: analysing samples in clusters, in the same order in which they were collected (e.g. by year). This approach may be used, for example, in physiological studies (e.g. Takizawa et al., 2004) and has the advantage that samples can be analysed immediately without any issues in placing or labelling of samples. However, sequential structuring of samples confounds cluster with organising variable (e.g. year) effects (Figure 7.2a). The second approach, randomisation of samples from multiple years within a cluster, ensures that samples are sufficiently mixed to avoid confounds, and should already be standard practice (Figure 7.2b). The use of randomisation is widespread in, for example, telomere length (e.g. Spurgin et al., 2017), disease (e.g. Swanson et al., 2015) and hormone analyses (e.g. Dantzer et al., 2013). However, this randomisation approach requires a delay before analyses can be completed, so that samples collected at different time points can be analysed together, and organising variable and cluster effects can be separated. Furthermore, the randomisation of large numbers of samples is time-consuming and detailed reordering of samples from the biobank is prone to error due to sample labelling and placing. Most importantly, however, is that after applying this randomisation approach once in a long-term study, any subsequently collected samples cannot be directly compared to the previously randomised samples as they will be subject to statistically inseparable variation due to clustering of the samples already analysed. For example, randomising two time periods of five years of sampling separately into two clusters results in uncontrollable variation between these two clusters and confounds the first five years in cluster one with the subsequent years in cluster two (Figure 7.2b), leading to cross-classified data structures (Figure 7.1c). Analysing the same samples multiple times in subsequent clusters can avoid this issue, often referred to as ‘golden’ or ‘reference’ samples. However, the additional costs or potential depletion of the ‘golden’

sample can make this approach difficult. More importantly, it is unclear how effectively one golden sample can control for among-batch and among-cluster variation. For example, the 'golden' sample might not be representative of all samples, and the sample can degrade over time thus not returning the same value in different analyses. In short, these two popular approaches to structuring cross-classified samples do not fully account for among-cluster and among-batch variation, leaving an unknown amount of variance unquantified and thus compromising conclusions drawn from such studies.

The analyses of longitudinal data can be turned into a nested design when samples from a single individual are aggregated within a batch and cluster (Figure 7.1a). This is thought to increase the statistical power to detect within-individual effects. The reasoning is that longitudinal samples are then exposed to the same technical noise, which allows greater statistical power to dissect out the biology from batch effects (Beirne et al., 2014; Herborn et al., 2014; Nettle et al., 2015; Pauliny et al., 2015; Rius-Ottenheim et al., 2012; Sudyka et al., 2014). Although the aggregation approach may be optimal for certain questions and data structures (e.g. experimental studies where the focus is on within-individual changes, while having controlled for many other sources of variation), the increasing application of physiological assays in long-term studies requires a different approach because the aggregation of longitudinal samples in a single batch has four disadvantages. First, analyses need to be postponed until all samples from a single individual have been collected. Second, aggregation requires detailed picking and reordering of samples, which increases the likelihood of human error, sample mix-ups and therefore false conclusions. Third, confounding variables that covary with the individual samples taken from one individual are not effectively separated from batch or cluster (e.g. seasonal effects). Fourth, it is rare for within-individual variation in a trait to be the sole interest, often between-individual variation is of interest too, and aggregating individuals within a batch could reduce the ability to estimate between-individual variation when individuals are aggregated in and thus

confounded with batch effects. Thus although assumed, it remains to be determined whether aggregation increases statistical power to detect within-individual effects so substantially that it would outweigh these four disadvantages. Hence, the approaches to structure samples for analyses in long-term studies suffer from confounding effects, cross-classified data structures and increased likelihood for human error, and cannot provide the comparable analyses of samples over time required in long-term studies.

Here, we present an approach to the analysis of samples from growing biobanks that, while maintaining statistical independence, accounts for among-cluster variation and controls for other potentially confounding effects (Figure 7.2c). Additionally, we provide a case study of this novel approach and subsequently test the assumption that aggregating longitudinal samples within batches results in greater statistical power to detect within-individual effects. We then discuss the analysis of long-term data and highlight the importance of statistical mixed models. While we will mainly consider the field of evolutionary biology, using telomere dynamics as an illustrative example, these considerations and techniques can be applied to a range of fields, including epidemiology, ecology and laboratory-based science.

## **7.3 Materials and Methods**

### **7.3.1 Slicing approach**

We have developed a slicing approach to structure samples from growing biobanks, such that recently collected samples are analysed in clusters together with previously obtained samples, ensuring statistical independence of collection time and cluster. This approach can overcome the experimental design and statistical issues with cross-classification and confounding variables in long-term studies (Gelman and Hill, 2006; Greenland et al., 1999; Schielzeth and Nakagawa, 2013), by bridging batches and clusters together. This allows for a structured and *a priori* separation of the variation of interest from confounding variables and

when combined with mixed models copes well with cross-classified data structures. The biobank is divided into slices (Figure 7.2c), where a slice reflects a group of collectively gathered samples (e.g. in the same year) analysed together. Samples from a slice can be sequentially allocated to batches and only need to be analysed once, with the benefit of needing less sample volume and less degradation of samples. Separate samples from the same slice can be analysed in different batches or clusters (Figure 7.2c), bridging batches and clusters together. Slicing uses a varying proportion of samples from each given sampling period (i.e. slices), sequentially analysed in a single cluster, to statistically account for temporal and cluster variation. Slicing therefore allows convenient sequential analysis while maintaining statistical independence.

Depending on the frequency at which new samples are obtained, the 'width' of the slices can be changed (Figure 7.2c). For example, low analysis frequency requires wider slices to account for among-cluster variation. This decision is directly related to the slicing 'angle' (Figure 7.2c), which determines the degree of independence of sampling year from cluster. For example, if there are environmental effects related to the collection time of samples, slicing samples by collection time (i.e. lower angle) removes possible confounds with cluster effects. For slicing to be effective across clusters it requires multiple years/cohorts to be present within a single cluster and at least one of those years/cohorts to be present in a different cluster, allowing statistical separation of among-cluster variation and confounding effects. Depending on slicing width and angle, a slice should cover approximately one-third of each batch, when slicing across three batches, with three separate slices covering the same batch (Figure 7.2c and see simulations). Such a strategy also naturally allocates samples of certain slices to batches in subsequent clusters, bridging clusters together (Figure 7.2c) and allowing control of among-cluster variation. As a general rule, when you have more confounding effects smaller slices (i.e. lower angle and smaller width) are required to be able to partition these confounding effects. Smaller slices lead to a greater statistical power to



separate potentially confounding effects within and between batches (as there are more slices within a batch and each slice occurs in more batches). Setting the slicing angle and width is a trade-off between statistical independence (assessing statistical power in the case of confounding effects) and the number of samples that remain unanalysed until the addition of newly collected samples. This latter point is a constraint, as the number of samples that can be analysed simultaneously will be reduced, if only slightly, by this approach. We argue that the creation of statistical independence and accounting for among-cluster variation are merits that outweigh this limitation.

### **7.3.2 A case study: structuring samples for telomere length analysis in wild house sparrows**

We provide a case study of how slicing can be applied to structure samples for analysis in a long-term (>20 years) study on a natural population of house sparrows (*Passer domesticus*) on Lundy Island, UK (Schroeder et al., 2015). House sparrows are a relatively long-lived species (on Lundy: mean lifespan is  $3.5 \pm 1.4$  SE, maximum lifespan is 9 years; Schroeder et al., 2012). The Lundy population has been systematically studied since 2000 and the adult population size varies between years (Simons et al., 2015). Immigration to and emigration from the island is low (0.5% of recruits; Schroeder et al., 2015), with an annual resighting probability of 0.91–0.96 (Simons et al., 2015). This closed island population on Lundy thus provides precise ages and life-history data for all individuals.

We use a subset of the Lundy dataset containing 12 years of data (2000–2011; Table S7.1), where the population consisted on average of 130 individuals that were blood sampled on average twice a year. The total biobank we selected for in this case study contains 2,733 samples from 515 individuals. The hypothesis to be tested is that telomere length and age are negatively associated within individuals, and therefore we will analyse all samples collected every 6 years (i.e.  $12/6 = 2$  clusters) with 12 qPCR-plates (i.e. batches) in each

cluster (Figure S7.1). Samples are analysed sequentially, where each sample is analysed once. A key consideration is to separate variation in sources of interest from confounding variables by analysing samples with different confounding effects in the same batch. This ensures that confounding effects (e.g. sampling year) are not fully confounded with attributes of batch. Slicing, where samples are sliced across batches within a cluster, can achieve such separation in combination with mixed models to statistically correct for known confounding effects (e.g. qPCR-plate).

In the Lundy sparrow example, we first determine the slicing width, which depends on the analysis frequency and number of samples collected in each year. The analysis frequency (i.e. 2 clusters) is relatively low which results in many sampling years within a cluster. The contribution of a confounding sampling year effect can be determined by comparison of within-year to between-year effects, which requires sufficient samples from a single year analysed in the same batch and therefore wider slices. However, the number of samples collected in each year varies markedly, resulting in a variable slicing width per year depending on the number of samples in each year (Figure S7.1). Secondly, we determine the slicing angle. Since the population density varied strongly between years, the slicing angle should be low (Figure S7.1). This way a single year crosses more batches which allows confounding effects (i.e. population density and year) to be separated from variation in sources of interest. Thirdly, since the number of samples exceeds the preferred slicing width and angle, multiple batches with the same lay-out will be used (Figure S7.1). These slicing parameters result in at least three slices within a batch to enable the separation of confounding environmental effects (e.g. population density, sampling year) from laboratory effects (e.g. batch), when using mixed models (Gelman and Hill, 2006).

The slicing approach allows an accurate estimation of the relationship between telomere length and age. Since the Lundy sparrow study is ongoing, the slicing approach can be continued into new clusters without inducing new confounding effects. For comparison,

the sequential approach would confound sampling year with batch effects while randomisation of samples could result in human and technical errors. Additionally, randomisation would not allow comparable analyses among clusters or flexible inclusion of current data in future analyses.

Effectively applying the slicing approach to one's own dataset thus minimally requires multiple slices within a batch and cluster, and at least part of one of these slices in another batch or cluster (Figure 7.2c; Figure S7.1). The slicing width can vary, for example, depending on the number of samples collected each year. Additionally, a low slicing angle is preferred since often there is a substantial number of confounding effects, and a lower slicing angle leads to slices crossing more batches and separation of confounding effects from variables of interest.

The benefit of the slicing approach over other strategies is that it allows convenient sequential analysis of the biobank and enables separation of variables of interest from confounding variables. However, the benefit of sequential analysis within the slicing approach disappears when samples from a single individual need to be aggregated within the same batch (e.g. Beirne et al., 2014; Nettle et al., 2015). We therefore determine whether aggregation of longitudinal samples from a single individual provides greater statistical power and precision in long-term studies to detect any within-individual, between-individual, year or cohort effects through simulations.

### **7.3.3 Simulations**

We used simulations run in R 3.3.1 (R Development Core Team, 2019) to determine the statistical power (i.e. ability to reject the null hypothesis when false) and precision (i.e. width of the distribution) to detect individual, year and cohort effects, using different sample allocation strategies (i.e. longitudinal samples aggregated in a single batch, randomly allocated to batches, or 'sliced' across batches).

We simulated a population of 200 individuals in 10 cohorts that were sampled once a year for a maximum of 5 years, providing an equal sample size in all simulations. ‘Telomere length’ was used as an example response variable; however, this is applicable to any longitudinally measured continuous variable. Starting telomere length was drawn from a Gaussian distribution to fix between-individual standard deviation (SD = 1.00) and all individuals shared the same within-individual shortening rate of telomeres (0.06\*1, scaled to SD = 1 parameter, = 0.06 per year).

Year effects were simulated by taking 0.7 multiplied by a generated value drawn from a uniform distribution (between 0 and 1) for each year and added these to the response variable. In separate simulations we replaced year with cohort effects (20 individuals per cohort) by taking 0.9 multiplied by a generated value from a uniform distribution (between 0 and 1) for each cohort. We chose to model ‘year’ and ‘cohort’ as possible biological confounds with experimentally induced variation. The choice to model such specific biology is rather arbitrary as we are simulating the confounding effect of ‘batch of analysis’ and biology. We also conduct additional simulations with varying strengths for year and cohort effects to determine the robustness of the results.

Individual probability of death was then modelled via telomere length associated with mortality (Eq. 1) as:

$$y_i = \beta^{(\alpha * x_i)} \quad (1)$$

where  $x$  is initial telomere length for  $i$ th individual, with a baseline probability of death ( $\beta$ ) of 0.25 and a slope ( $\alpha$ ) of -0.23, providing mortality risk ( $y_i$ ) per year. This resulted in the probability of death varying with  $\pm 2$  SD telomere length from 0.14 to 0.36 per year. Death for each simulated individual was determined by drawing from a uniform distribution (ranging 0 to 1) to determine a simulated death. Mortality was partly determined by the response variable (to simulate selective disappearance from the population, determined by the between-individual age component, see next paragraph), with variable telomere lengths

to start with (between-individual variation) and a set within-individual shortening (within-individual age component, see next paragraph).

We simulated the relationship between telomere length and age (in years) both within and between individuals. Between-individual effects were modelled using the mean age at which the individual's trait was measured, and within-individual effects as the age at which an individual's trait was measured minus the mean measurement age for that individual (van de Pol and Wright, 2009).

Simulations were run 5,000 times, for a varying number of samples (12, 24, 36, 48) per batch and simulated differences between batch means (batch attributable error, SD: 1, 2.5, 5, 10, 20, 40). This error is relatively high to ensure we control for potential effects of batch attributable error when determining the variation in statistical power among sample allocation strategies. Simulations were repeated three times to obtain three separate results per sample allocation strategy.

The slicing strategy was simulated at an angle that resulted in at least three slices per batch. Note, to start the sample allocation, the first batch was filled by 3/4 with the first slice and by 1/4 with the second slice, where subsequent batches were filled by 1/4, 1/2 and 1/4 with subsequent slices (Figure 7.2c). Additional simulations were run with the slicing angle halved, slicing width halved, and a doubled sample size ( $n = 400$ ).

The simulated data were analysed using linear mixed models in *lme4* 1.1-14 (Bates et al., 2015), where the model included random effects (at the intercept level) for individual (to control for repeated measurements on the same individual) and batch, and year or cohort was fitted as a fixed factor. Statistical power was determined by the number of significant values ( $p < 0.05$ ) for each variable out of the total number of simulations ( $n = 5000$ ). It is important to understand the effect of sample allocation strategy on precision estimates, as well as statistical power. We therefore quantified precision as the width of the distribution of parameter estimates from the models run on the repeated simulated datasets, as the

absolute difference between the 75% and 25% percentile divided by the median (note, a precision value closer to zero means higher precision).

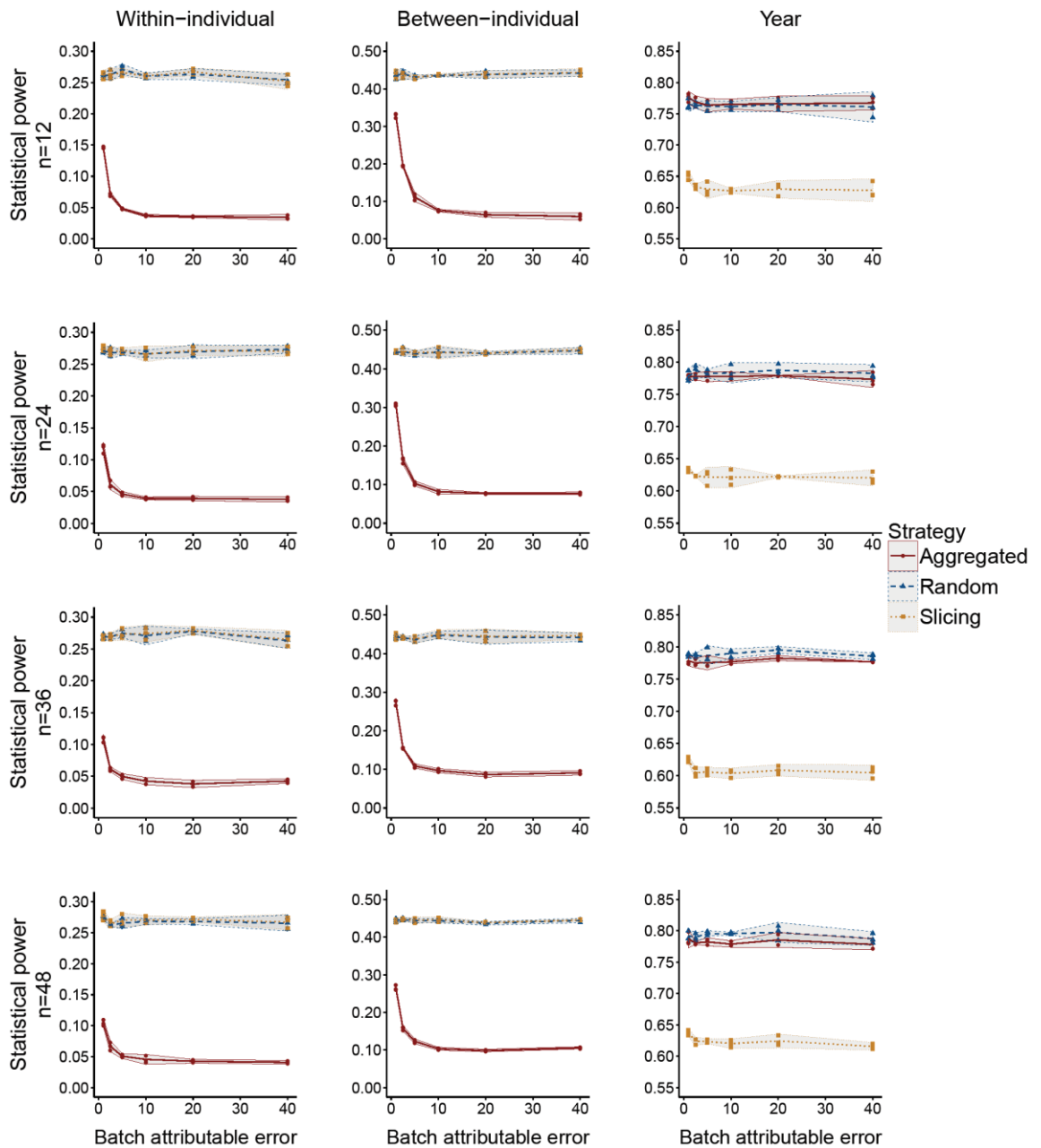
Parameters of the simulations were manually optimised so that a statistical power of approximately 0.5 was achieved to detect between-individual effects for the random allocation strategy, determined by a t-value of less than -2 ( $\alpha \approx 0.05$ ). This intermediate level of statistical power avoids thresholding effects at either end of the power spectrum (0 or 1). Such a simulation strategy maximises the sensitivity in detecting any modulation in relative statistical power among sample allocation strategies, which is our focus rather than an achieving a certain absolute statistical power.

#### **7.4 Results**

Our simulations tested the widely held assumption that aggregating longitudinal samples of the same individual in a single batch increases statistical power to detect within-individual effects (e.g. Herborn et al., 2014; Nettle et al., 2015). In simulations with year effects, the statistical power to detect within-individual effects was much lower when longitudinal samples were aggregated (mean statistical power $\pm$ SD across sample sizes and three runs per simulation = 0.059 $\pm$ 0.030) than when samples were sliced across batches (0.269 $\pm$ 0.008) or randomly allocated to batches (0.267 $\pm$ 0.007; Figure 7.3). For between-individual effects, again, the statistical power was much lower when longitudinal samples were aggregated in a single batch (0.138 $\pm$ 0.077) compared to when samples were sliced across batches (0.443 $\pm$ 0.007) or randomly allocated to batches (0.441 $\pm$ 0.007; Figure 7.3). The statistical power to detect year effects was higher when longitudinal samples were aggregated in a single batch (0.776 $\pm$ 0.008) or randomly allocated to batches (0.782 $\pm$ 0.014) than when sliced across batches (0.622 $\pm$ 0.012; Figure 7.3). However, a lower slicing angle (crossing four batches; 0.741 $\pm$ 0.009) and smaller slicing width (half a batch; 0.751 $\pm$ 0.007) resulted in a similar statistical power to detect year effects to aggregation of longitudinal samples and

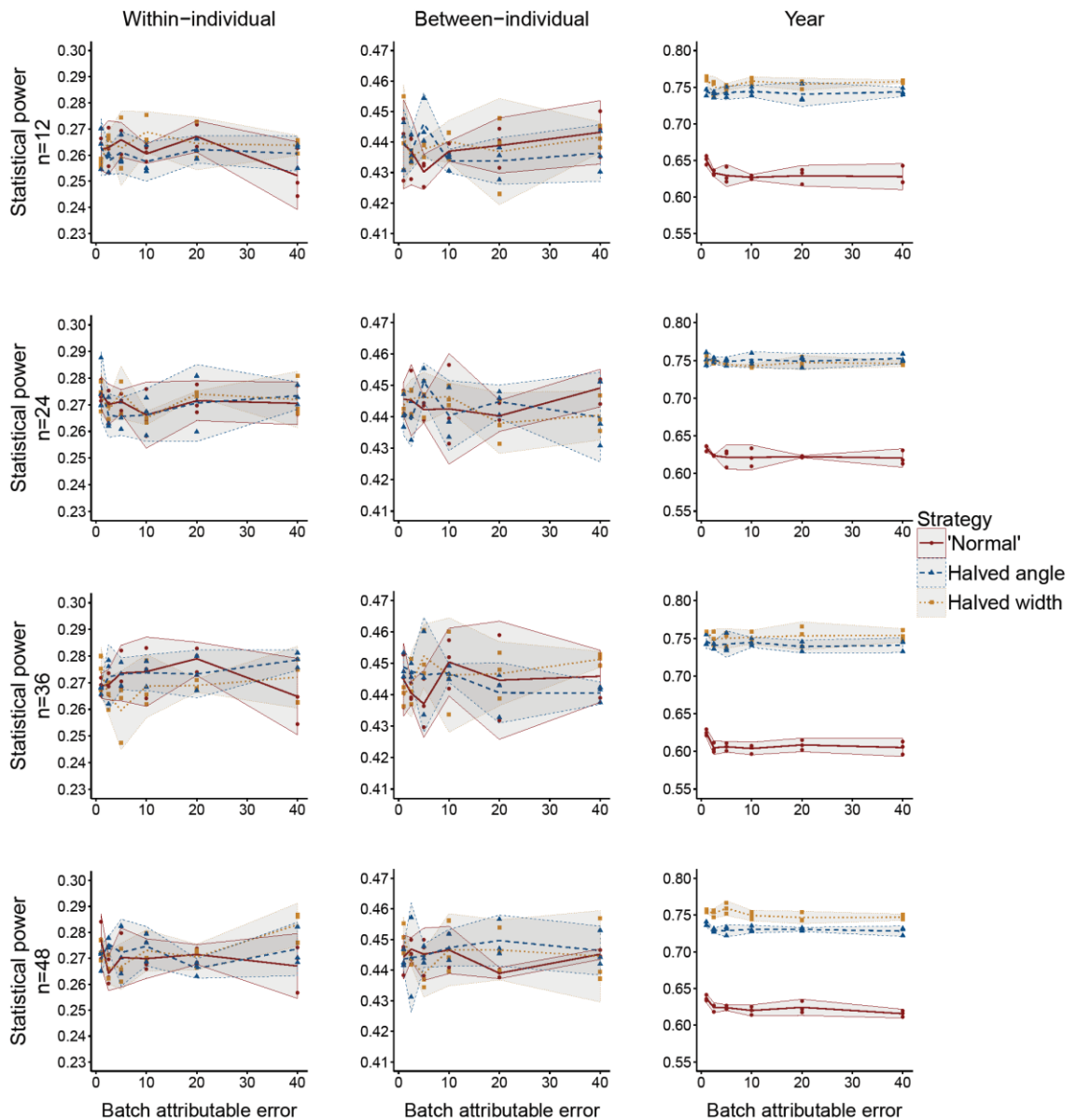
random allocation, while maintaining statistical power to detect within- and between-individual effects (Figure 7.4).

In simulations with cohort effects, the statistical power to detect within- and between-individual effects was lower when slicing across batches ( $0.159 \pm 0.032$ ;  $0.324 \pm 0.020$ ) compared to aggregation ( $0.557 \pm 0.009$ ;  $0.390 \pm 0.020$ ) and randomisation ( $0.542 \pm 0.014$ ;  $0.423 \pm 0.008$ ) approaches (Figure S7.2). However, statistical power to detect cohort effects was greater for slicing ( $0.413 \pm 0.009$ ) and randomisation ( $0.462 \pm 0.008$ ) compared to aggregation of longitudinal samples in a single batch ( $0.142 \pm 0.044$ ; Figure S7.2). A doubled sample size ( $n = 400$ ), either for simulations with year or cohorts effects, increased statistical power but did not alter variation in statistical power among sample allocation strategies (Figure S7.3–S7.4). Additionally, varying the strengths of year and cohort effects changed the statistical power, but not the variation among sample allocation strategies (Figure S7.5–S7.8).



**Figure 7.3: Statistical power analyses of simulated data for individual and year effects among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between year, within- and between-individual effects.**





**Figure 7.4: Statistical power analyses of simulated data for individual and year effects among four batch sizes ( $n = 12$  to  $48$ ) using three different slicing parameters: (1) slicing angle that crosses two batches with a slicing width of a single batch (solid, red), (2) halved slicing angle which crosses four batches (dashed, blue) or (3) halved slicing width of half a batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between year, within- and between-individual effects.**

In simulations with either year or cohort effects, the precision to estimate within- and between-individual effects followed similar patterns to statistical power in the respective simulations, with greater precision for the approaches that showed greater statistical power

(Figure S7.9–S7.10). However, precision to estimate cohort and year effects was the opposite of statistical power, where approaches with lower statistical power showed greater precision to detect such effects (Figure S7.9–S7.10). A doubled sample size ( $n = 400$ ) increased precision but did not alter variation in precision among sample allocation strategies (Figure S7.11–S7.12). Additionally, varying the strengths of year and cohort effects changed the precision, but not the variation among sample allocation strategies (Figure S7.13–S7.16).

Our slicing method performs similar to randomisation of samples and outperforms aggregation of longitudinal samples to disentangle within- and between-individual effects when year effects apply, an objective shared by many longitudinal studies (Nussey et al., 2013; van de Pol and Wright, 2009). Simulations were run for a wide range of parameters and sample sizes (Figure 7.3, 7.4, S7.2–S7.16). When desirable, different parameter sets specific to current or future datasets can be included in the script provided.

## **7.5 Discussion**

The analysis of comprehensive long-term datasets is often complex due to cross-classified data structures and difficulties in separating variation in sources of interest from confounding variables, such as separating year from batch effects. Our simulations clearly demonstrate that statistical power was greater for within- and between-individual effects when samples were randomised or sliced across batches when year effects apply. The reduction in statistical power for aggregation of longitudinal samples in a single batch to detect such effects can be explained by the confounding of the year and batch effect. Conversely, in simulations with cohort effects there was greater statistical power for within- and between-individual effects when samples were aggregated within a single batch or randomised, compared to slicing across batches. The lower statistical power for slicing to detect such effects is the consequence of a low number of cohorts in our simulations, where cohorts are sequentially ordered instead of mixed among slices, which results in confounding

effects between cohort and batch within the slicing approach. A higher number of cohorts in the simulations will lead to a mixture of cohorts among slices and result in similar statistical power to detect within- and between-individual effects for all three sample structuring strategies. This highlights the importance choosing appropriate slicing angles and widths, ensuring adequate variation of potential confounds (e.g. cohort/year) in a single batch.

The greater statistical power to detect within-individual effects for slicing and randomisation when year effects apply was the consequence of appropriate statistical methodology, accounting for batch, individual and year through fixed and random effects. These results disprove the assumption that samples from a single individual need to be analysed in the same batch for greater statistical power to detect within-individual effects (e.g. Beirne et al., 2014; Nettle et al., 2015). Such efforts will reduce the statistical power of the study and generate unnecessary effort in picking specific samples, which increases the likelihood of technical errors (e.g. sample mix-ups, freeze/thawing effects, transcription or pipetting errors).

The statistical power to detect year effects was greater when samples were aggregated in a single batch or randomised across batches compared to slicing. However, when the slicing angle and width decreased there was no difference in statistical power to detect year effects compared with aggregation and randomisation approaches. This is because a lower slicing angle and smaller width reduces the confound between batch and year, as a slice crosses more batches or there are more slices per batch. In contrast, the notion that longitudinal samples should not be aggregated in the same batch becomes particularly pronounced when cohort effects occur. The effort of grouping samples from a single individual together collects cohorts together (an individual's cohort is fixed) in a batch thus reducing the statistical power to distinguish between different cohorts, even though this increases precision. Random allocation of samples and slicing have a substantially greater statistical power to detect cohort effects due to a higher mixture of cohorts within

the same batch. For telomere biology especially, estimating cohort effects reliably is important as it can affect telomere length strongly (Spurgin et al., 2017), but cohort effects are not always estimated. All these results are robust against a variety of batch errors, sample sizes and strengths of year and cohort effects.

#### *Integral approach to growing biobank analysis*

The optimal sample structuring strategy for analysing long-term datasets depends on the structure of the data and questions of interest. However, in the majority of long-term datasets, slicing has benefits over other structuring strategies by overcoming problems with confounding variables and cross-classified data structures which commonly occur in the analysis of long-term studies.

The assumption that longitudinal samples should be aggregated in a single batch could hinder the slicing approach, but our simulations have disproven this assumption. Slicing performs, in terms of statistical power and precision, equally well to randomisation when applying correct slicing parameters (i.e. low width and angle). Slicing across batches and clusters and bridging them together provides the slicing approach with statistical power to disentangle confounding effects.

The key benefit of slicing over randomisation is that slicing allows separate analysis of current data and flexible inclusion of these data into future analyses without completely confounding the analysis. Furthermore, slicing allows sequential analysis of samples, which only need to be analysed once, preventing complicated sample labelling and placing among clusters, reducing sample volume required and avoiding any defrosting issues and therefore reducing the potential for human error.

Slicing has some potential limitations. For example, substantial differences among years in the number of samples collected could limit the ease with which the slicing approach is applied. Additionally, a failed analysis of samples (e.g. plate failure leading to sample loss

during analysis) using slicing results in missing data within a certain time window, whereas with randomisation this is scattered across the dataset. While slicing performs similarly to randomisation in terms of statistical power and precision, we think that slicing is more practical with merits (i.e. sequential analysis, statistical independence) that outweigh the limitations. We stipulate that, because of sequential analysis in our slicing approach, hypotheses need to be pre-defined and power analyses conducted before experimental and statistical analysis (Fraser et al., 2018 and references therein).

The use of mixed models is common in the analysis of longitudinal datasets, especially in ecology (Bolker et al., 2009; Gelman and Hill, 2006). We highlight the use of mixed models because they are necessary when using the slicing approach to account adequately for experimental and environmental variation. The combination of slicing and mixed models in long-term studies allows analysis of commonly occurring cross-classified data structures that arise due to hierarchical biology mixed with cross-classified data collection and analysis. Interpretation of the variance components in these models depends on a crossed or nested design (Schielzeth and Nakagawa, 2013), where the random effect structure can be used to account for potentially confounding experimental and environmental variables with cluster effects (e.g. storage duration, batch). The failure to include these effects can inflate type I and type II errors when there is a temporal, spatial or other spurious correlation with any independent variable.

## **7.6 Conclusions**

A major current challenge in long-term studies is analysing data as it is collected while also including it in future analyses without creating uncontrollable variation, allowing comparison of results over multiple years or even decades. This requires the ability to compare differentially timed analyses that are potentially biased by confounding cluster effects. Our study shows the importance of considering the structure of samples among clusters and

batches in long-term studies. Our slicing approach retains statistical independence and accounts for among-cluster variation in the sequential analysis of growing biobanks. Slicing also provides similar statistical power and precision to detect cohort, year, within- and between-individual effects to randomisation, if analysed using appropriate statistical mixed models and consistent methodology to control for confounding effects. A single sample's scientific value increases through this approach, as it can be used separately in current studies, but can also be included in subsequent studies, providing sustainable (re)use of collected data. The approach we propose here (slicing and mixed models) is easy to apply and improves the potential for these growing biobanks to address important ecological and evolutionary questions.

# Supplemental information Chapter 7: Slicing: a sustainable approach to structuring samples for analysis in long-term studies

## Case study Lundy sparrows

**Table S7.1: Number of samples collected in each year in the subset of the Lundy sparrow study which are used to estimate telomere length.**

Year	Number of samples	Year	Number of samples
2000	270	2006	141
2001	282	2007	138
2002	288	2008	297
2003	117	2009	303
2004	168	2010	66
2005	279	2011	333

Samples are analysed on 96-well plates. Since the population density varied quite strongly among years, the slicing angle should be relatively low to ensure that a single year (i.e. slice) crosses many batches which allows separation of variables of interest from potential confounds (e.g. population density and year). The slicing width depends on the analysis frequency which is relatively low. However, although the number of samples collected in each year is high there is substantial variation between years, therefore the width of each slice also depends on the number of samples collected. Moreover, because the number of samples collected per year is high, and exceeds the sample size of a batch, it is advised to spread samples from a slice over multiple batches with the same lay-out to allow for sequential analysis of samples while maintaining statistical independence.

Slicing sparrow dataset

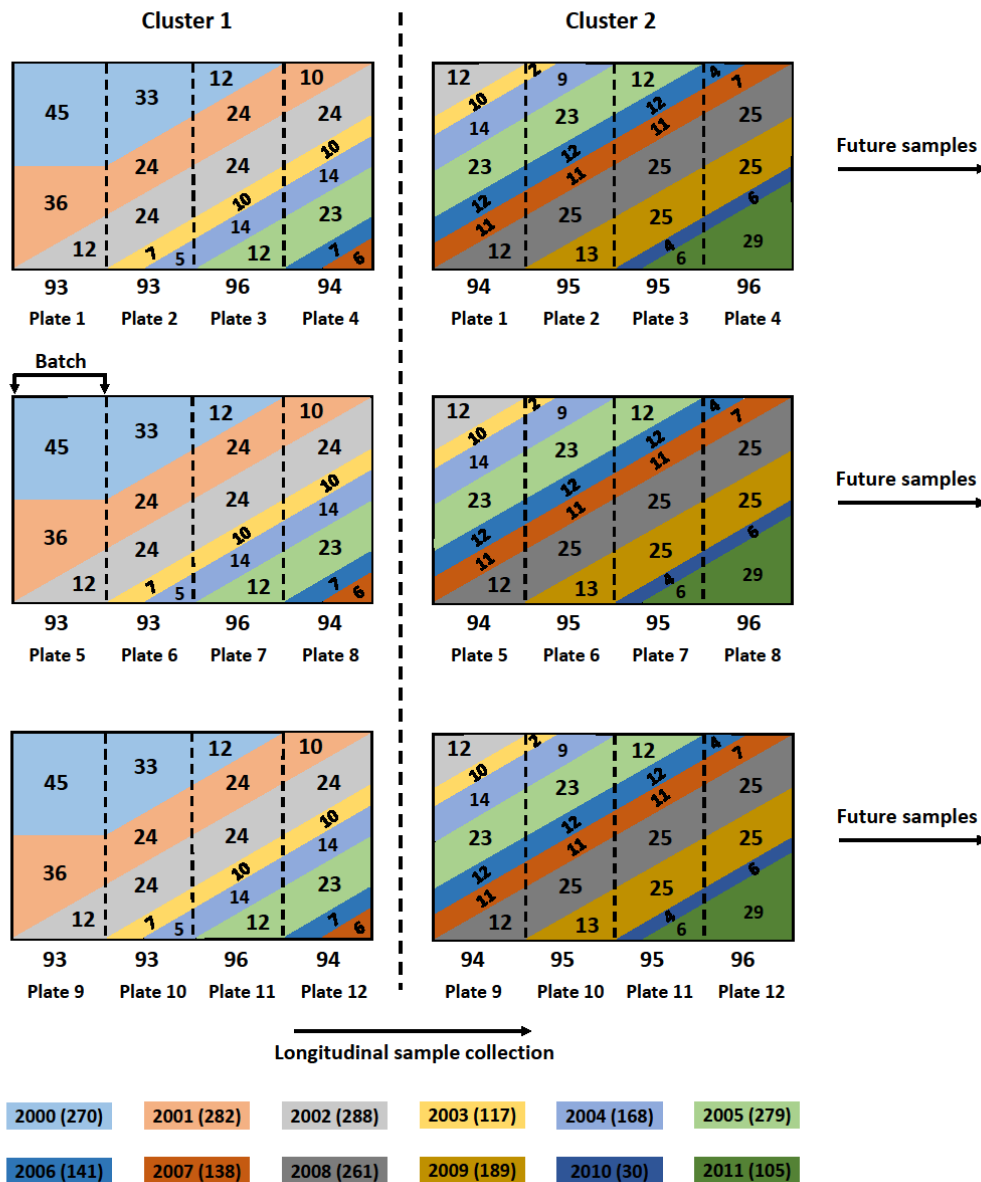
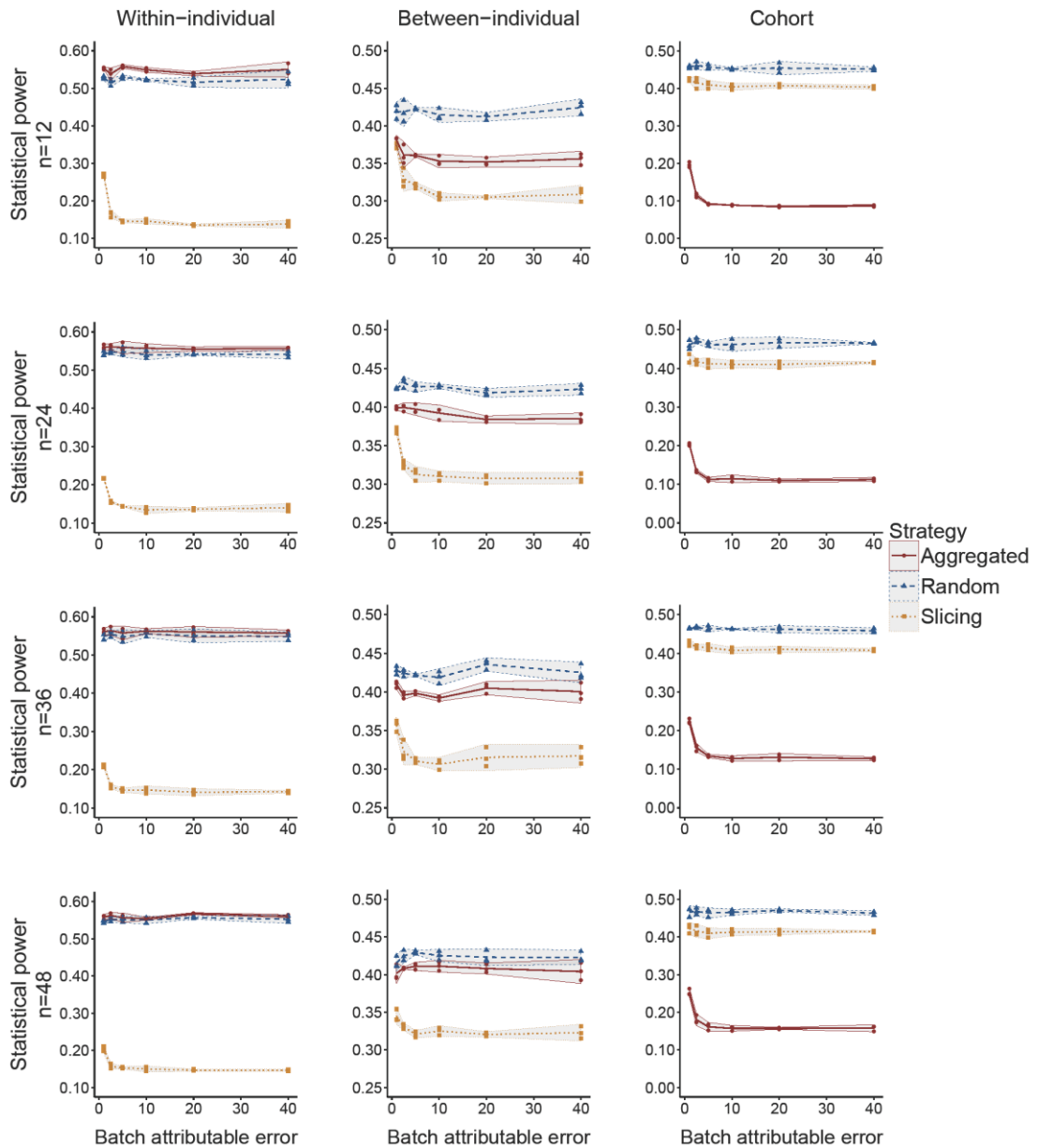


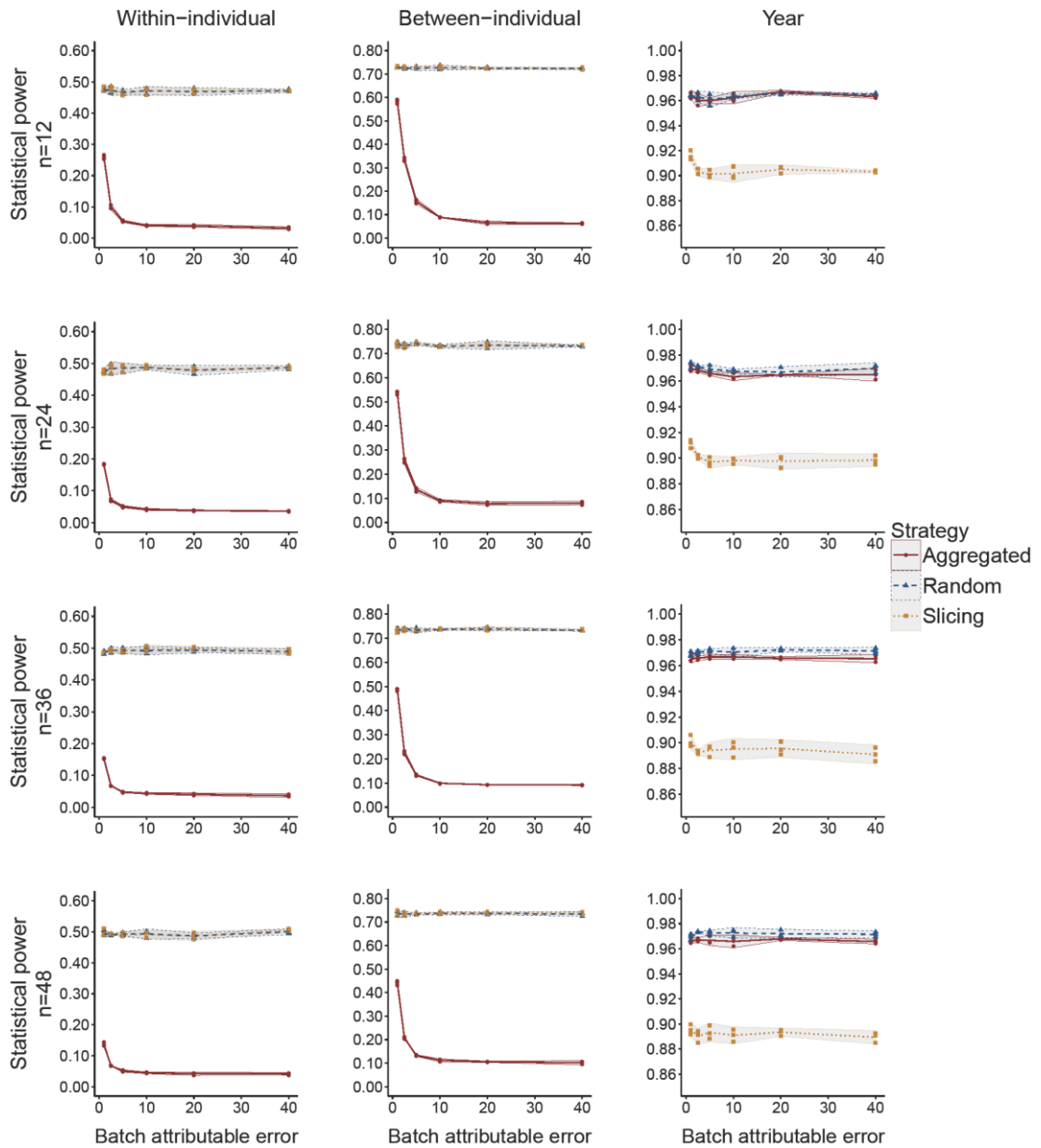
Figure S7.1: Suggested slicing parameters for the subset of the Lundy sparrow dataset. Each cluster consists of 12 batches (i.e. 96-well qPCR plates), where 3 qPCR plates have the same lay-out (i.e. 3 rows) because the number of samples exceed the preferred slicing width. Slicing width depends on the number of samples collected per year, whereas the slicing angle is low to avoid confounds with population density. The first and second slice have an alternative 'slicing pattern' because all samples from these first two years need to be analysed in the first cluster to avoid complicated sample placing in later analyses. Numbers in batches reflect the number of samples analysed per slice per qPCR-plate. The total number of samples on a qPCR-plate are shown below the qPCR plate. Colours of the slices correspond to a year in the legend at the bottom of the figure, which in brackets shows the total number of samples for that year analysed in these two clusters. All samples from the first 8 years are analysed by this approach, but not all samples from the last four years as



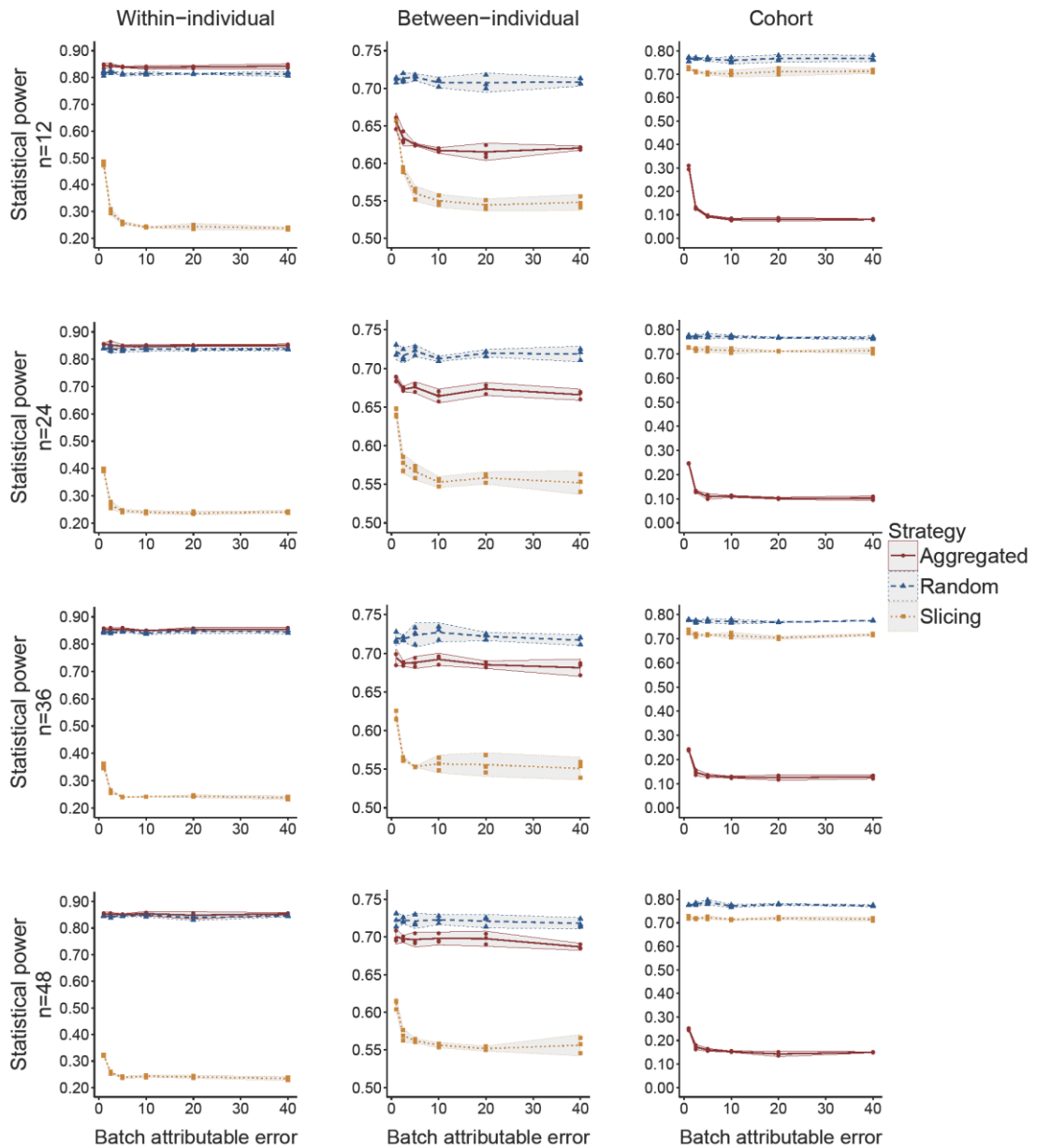
part of the biobank needs to stay in place to avoid confounds in future analyses. Running samples in duplicate or triplicate would mean half or a third of the samples of each slice per batch, but twice or three times the number of batches (with the same lay-out) per cluster, respectively.



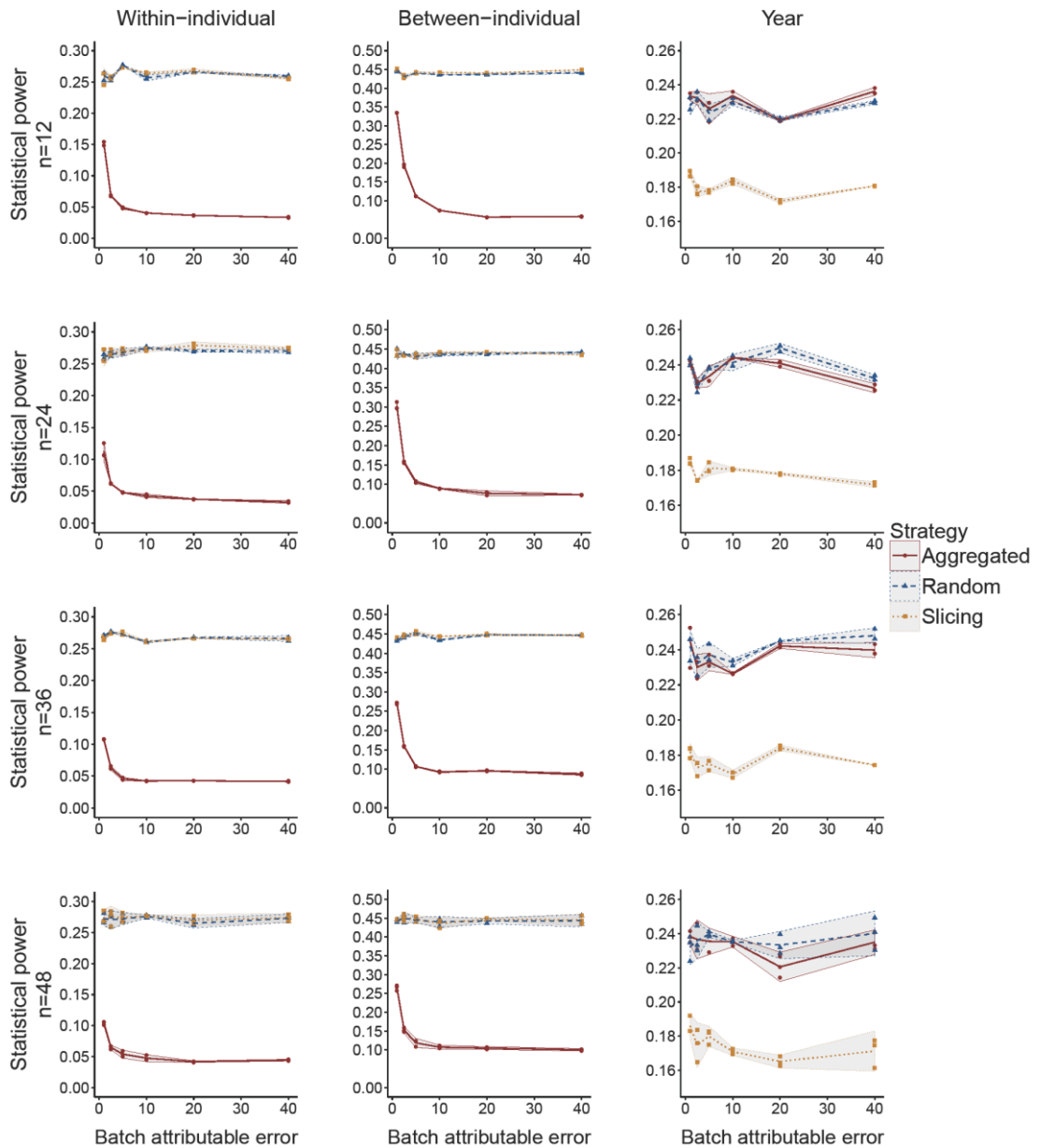
**Figure S7.2: Statistical power analyses ( $n = 200$ ) for individual and cohort effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between cohort, within- and between-individual effects.**



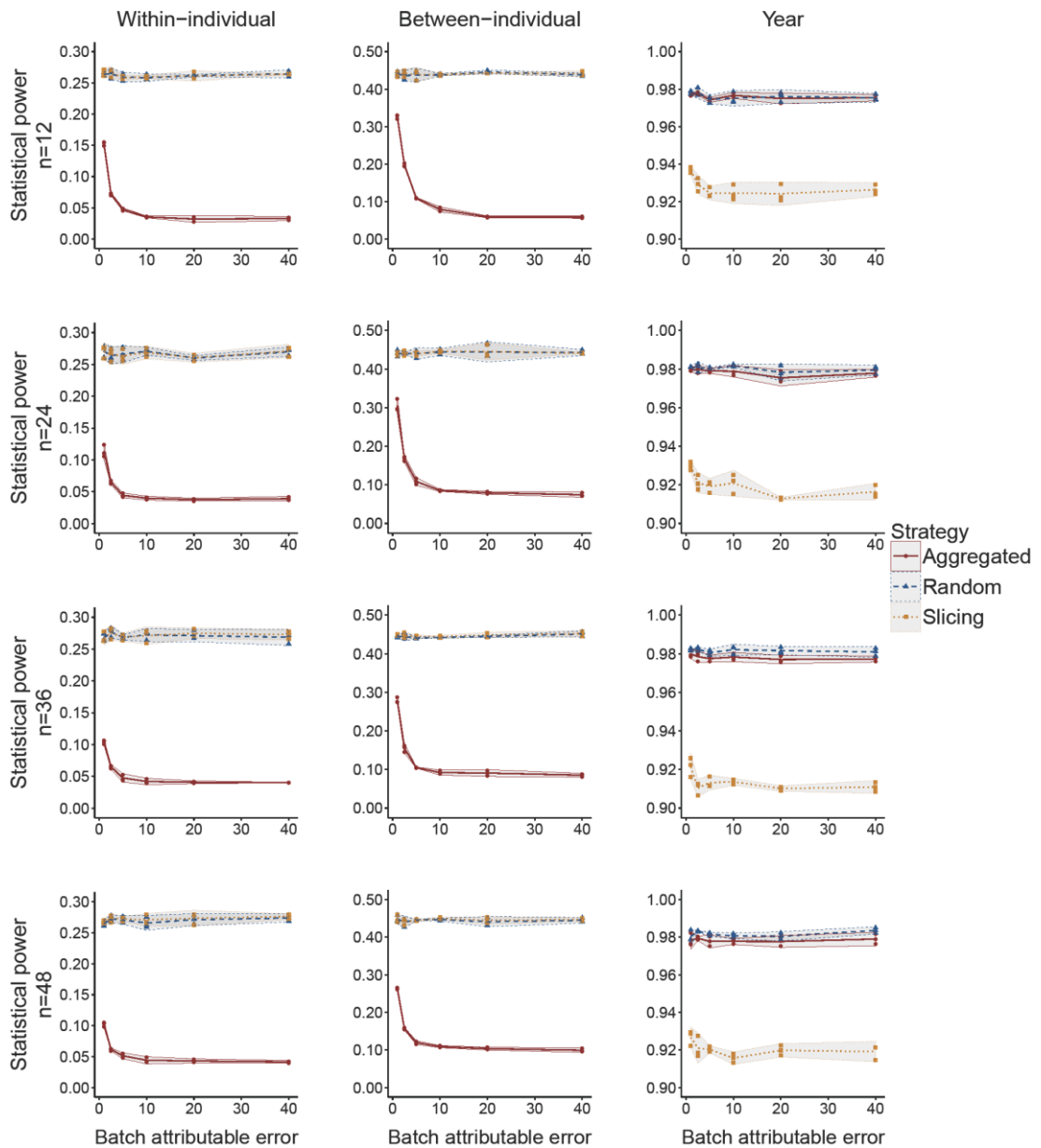
**Figure S7.3: Statistical power analyses ( $n = 400$ ) for individual and year effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between year, within- and between-individual effects.**



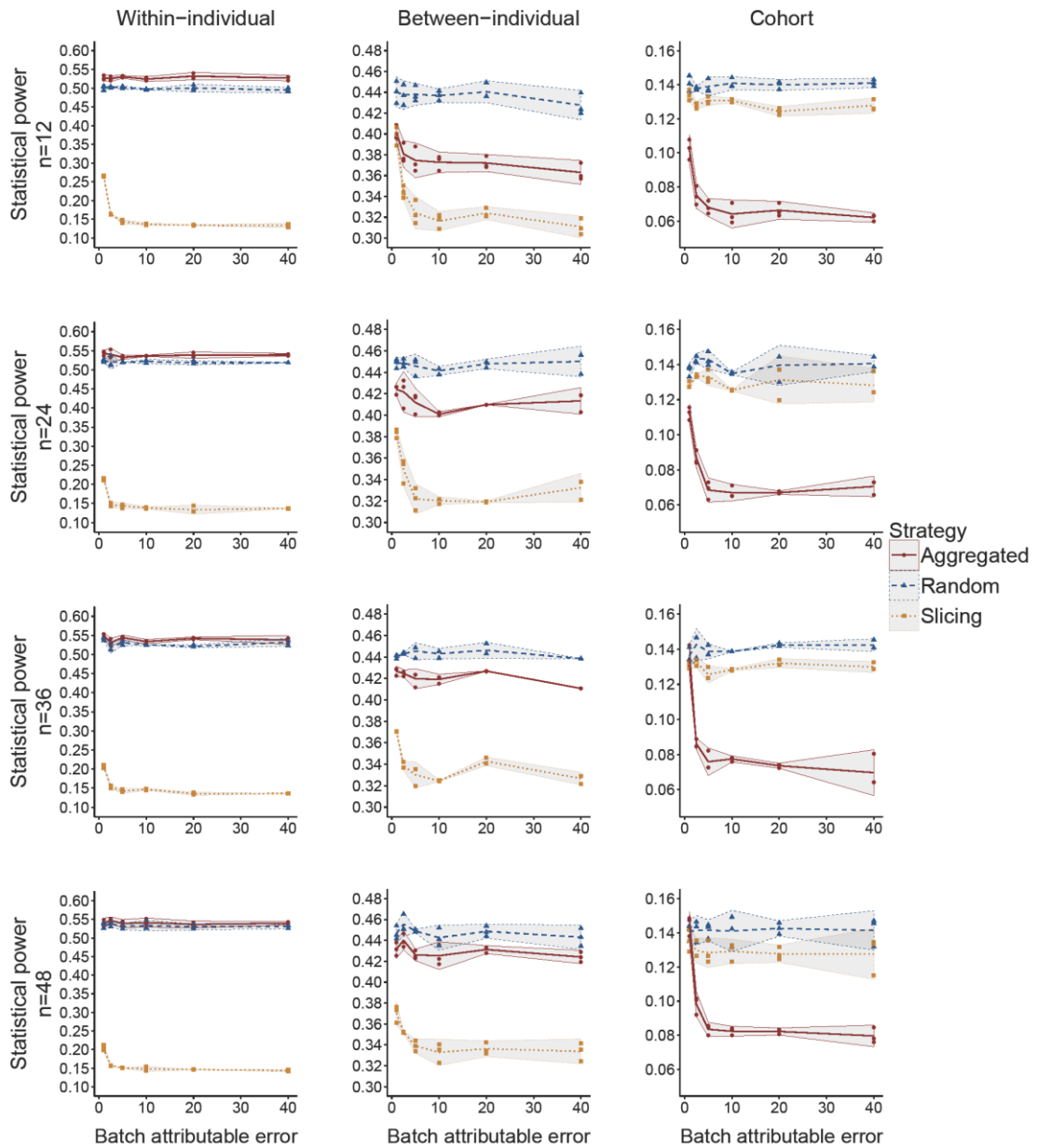
**Figure S7.4: Statistical power analyses ( $n = 400$ ) for individual and cohort effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between cohort, within- and between-individual effects.**



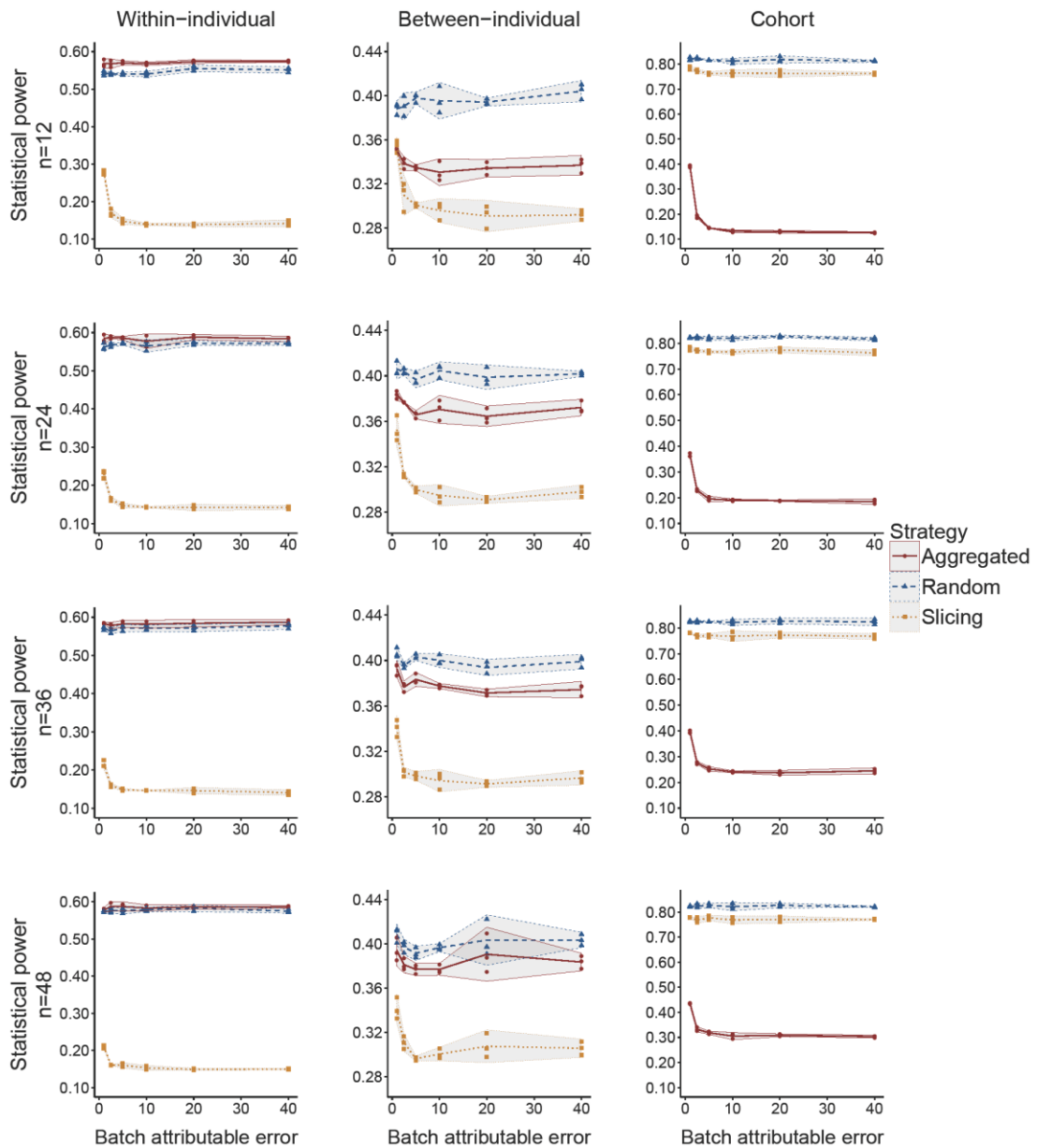
**Figure S7.5: Statistical power analyses ( $n = 200$ ) for individual and year effects (max. 0.35) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between year, within- and between-individual effects.**



**Figure S7.6: Statistical power analyses ( $n = 200$ ) for individual and year effects (max. 1.05) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between year, within- and between-individual effects.**

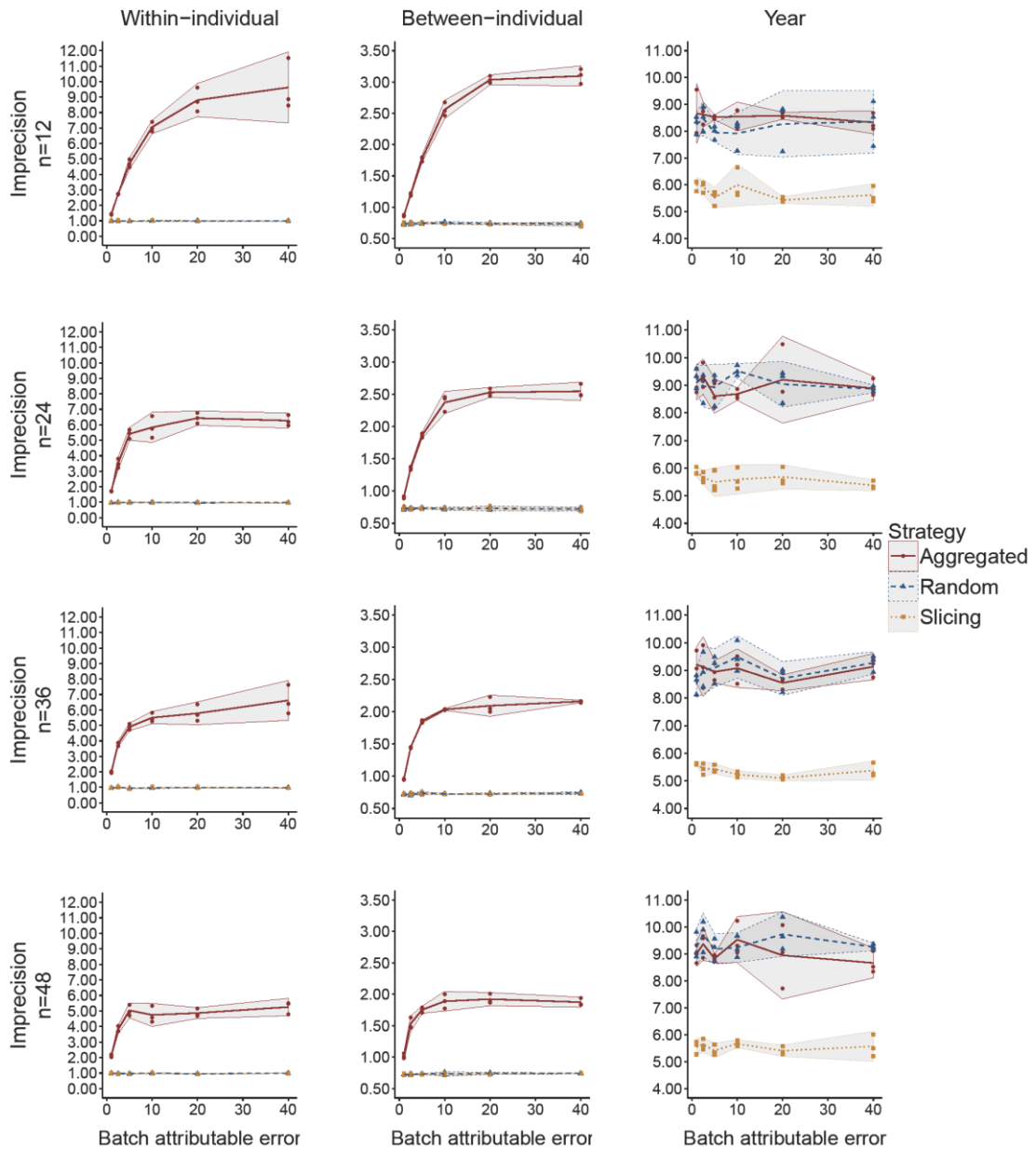


**Figure S7.7: Statistical power analyses ( $n = 200$ ) for individual and cohort effects (max. 0.45) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between cohort, within- and between-individual effects.**

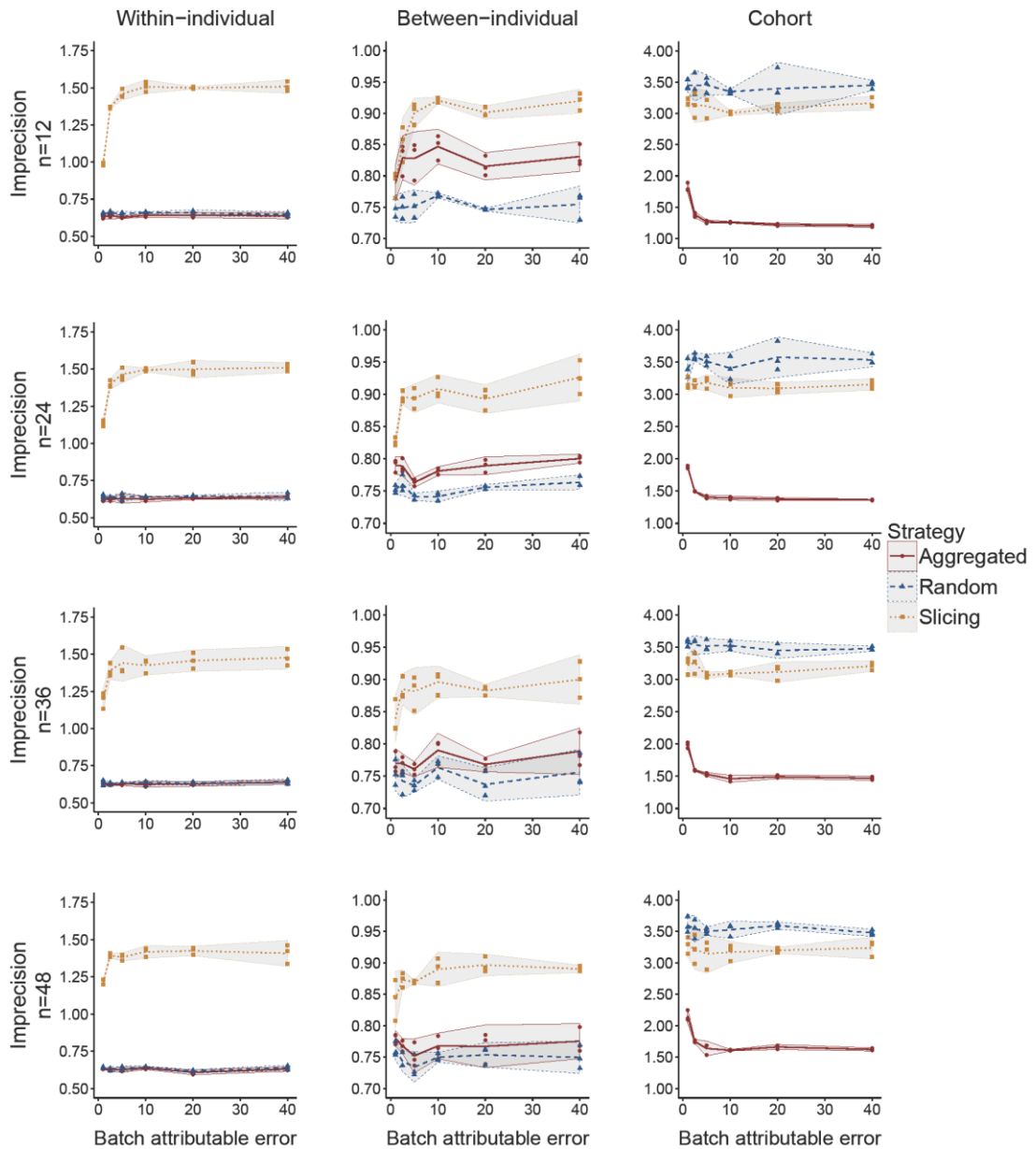


**Figure S7.8: Statistical power analyses ( $n = 200$ ) for individual and cohort effects (max. 1.35) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean statistical power per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Scales differ between cohort, within- and between-individual effects.**

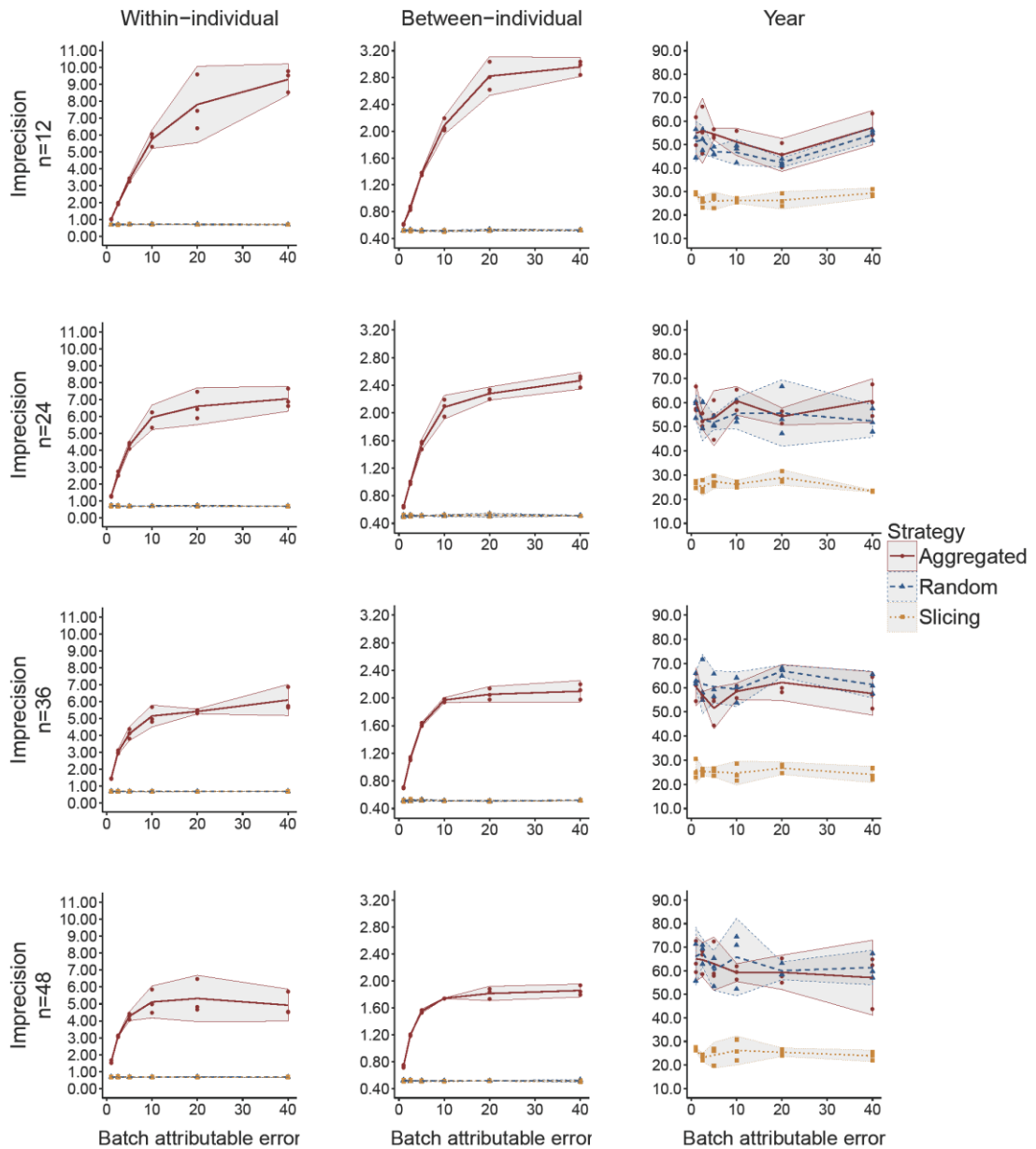




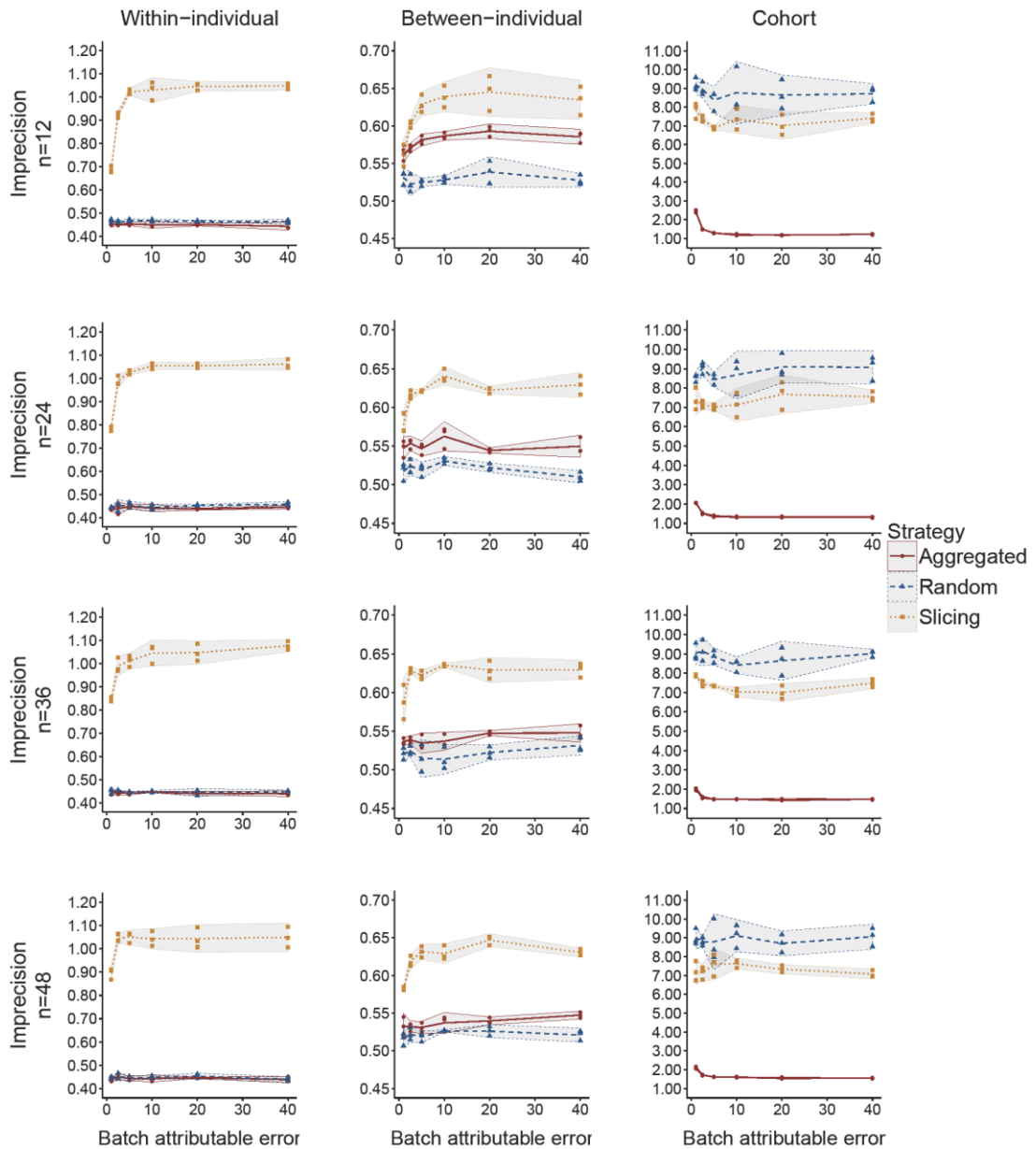
**Figure S7.9: Precision analyses ( $n = 200$ ) for individual and year effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between year, within- and between-individual effects.**



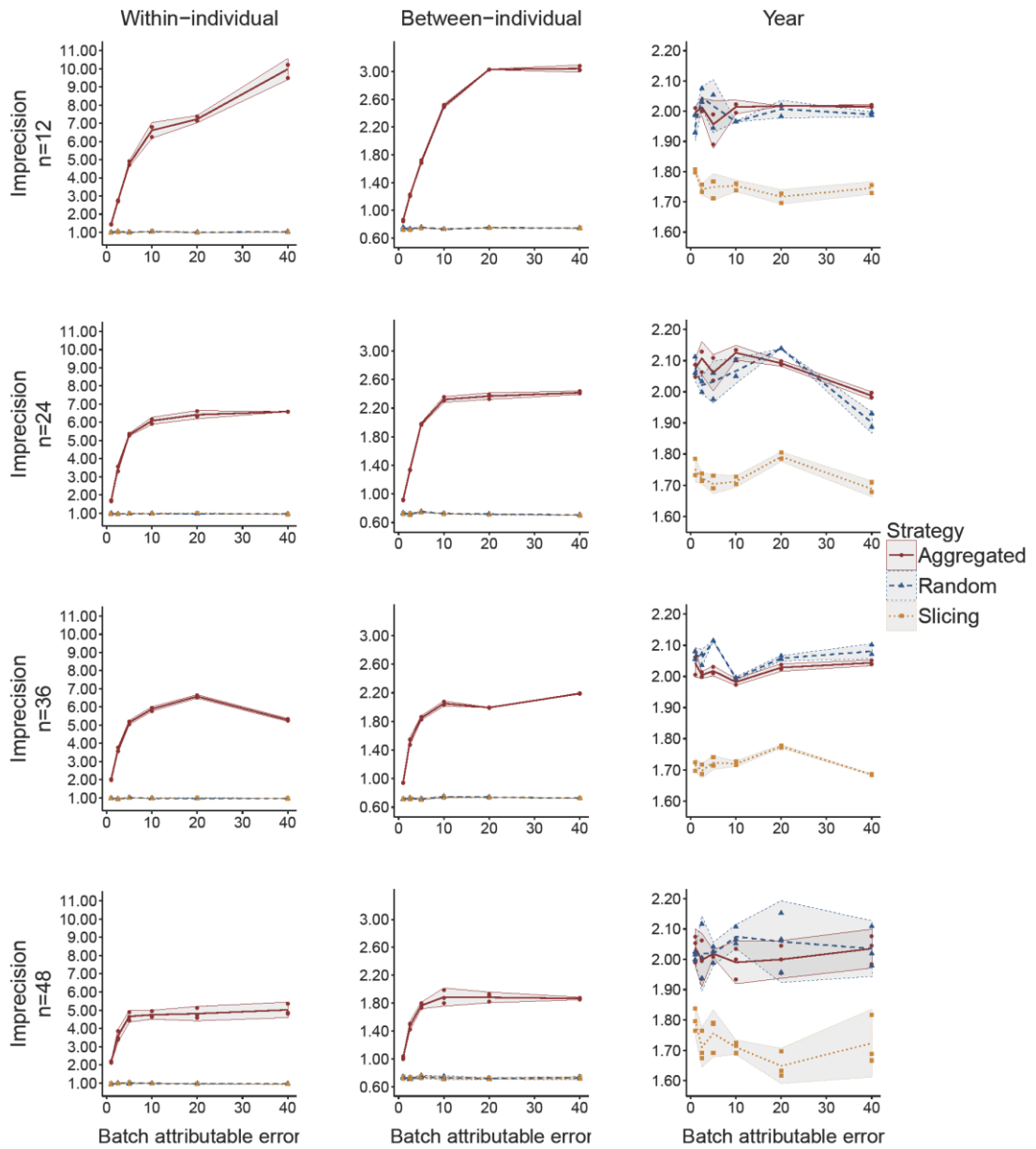
**Figure S7.10: Precision analyses ( $n = 200$ ) for individual and cohort effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between cohort, within- and between-individual effects.**



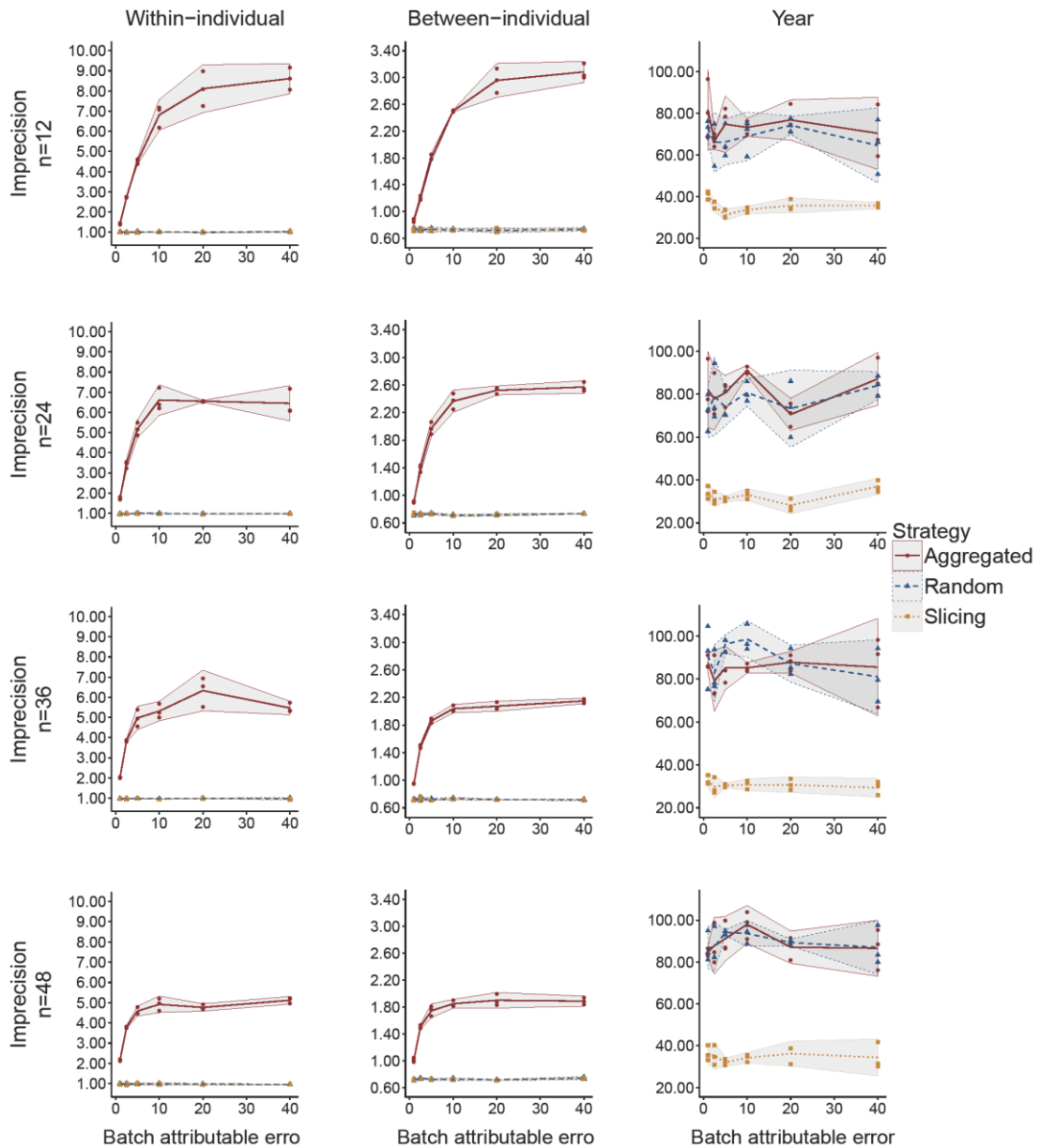
**Figure S7.11: Precision analyses ( $n = 400$ ) for individual and year effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between year, within- and between-individual effects.**



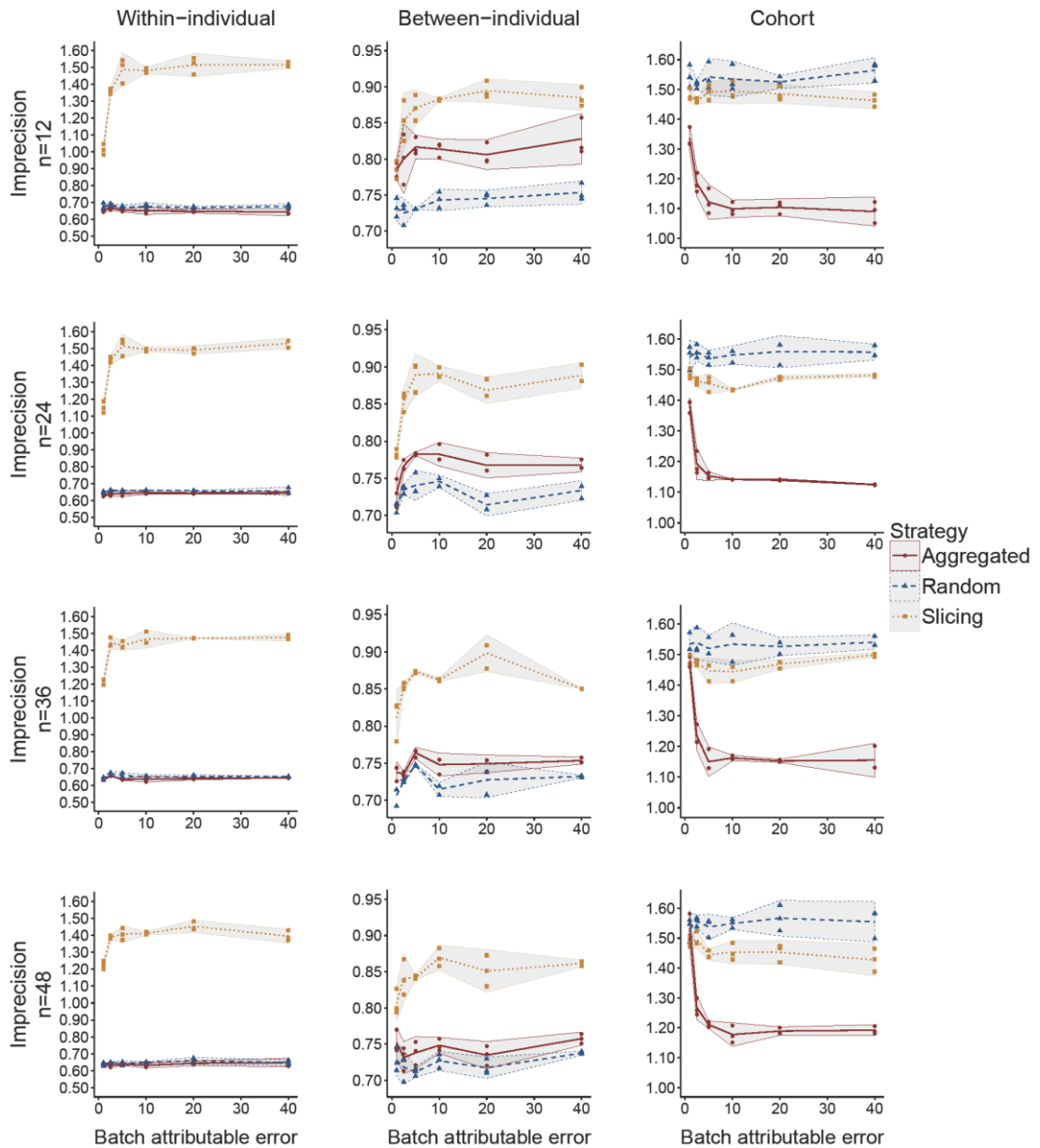
**Figure S7.12: Precision analyses ( $n = 400$ ) for individual and cohort effects among four batch sizes ( $n = 12$  to  $48$ ) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between cohort, within- and between-individual effects.**



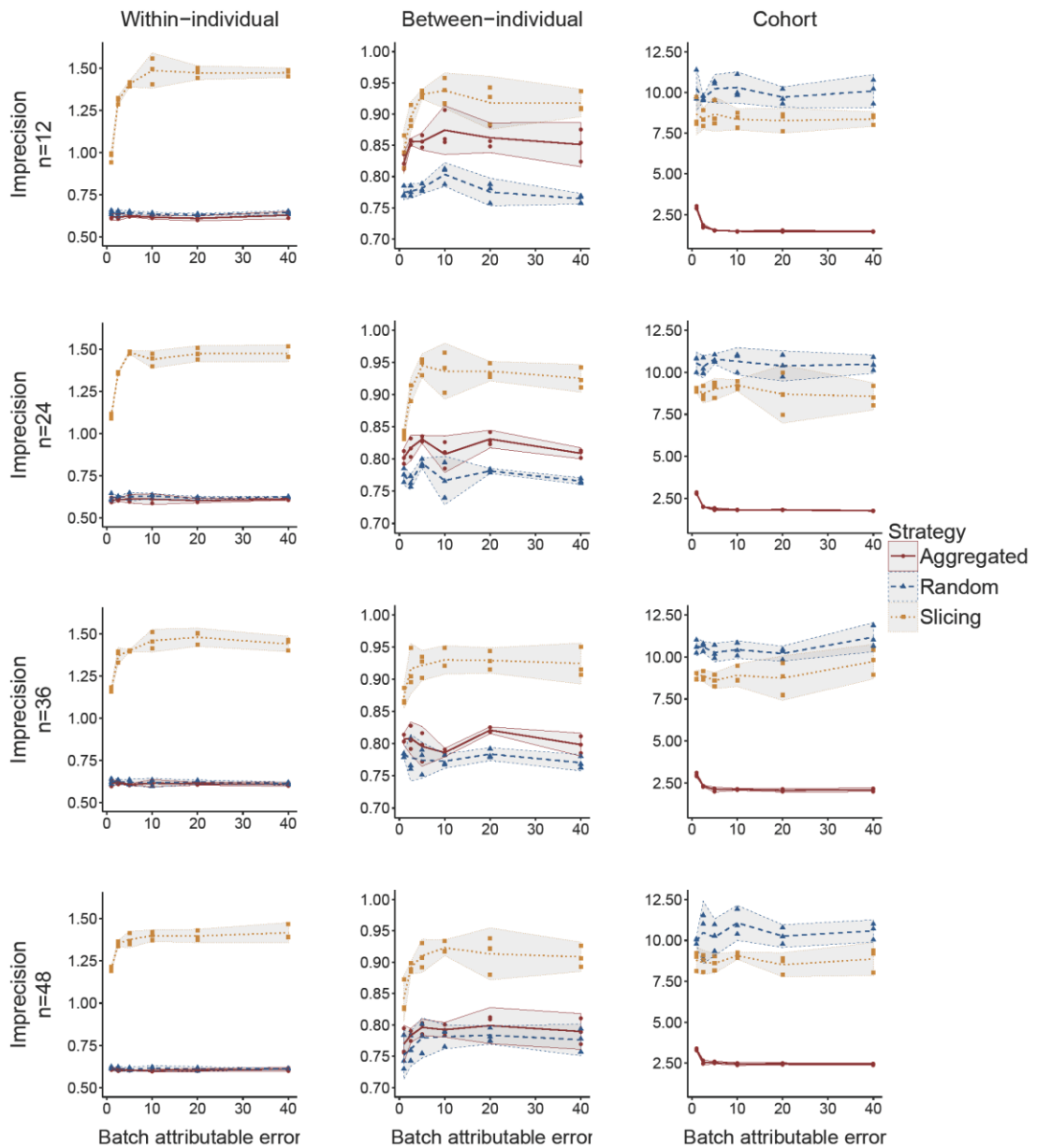
**Figure S7.13: Precision analyses ( $n = 200$ ) for individual and year effects (max. 0.35) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between year, within- and between-individual effects.**



**Figure S7.14: Precision analyses ( $n = 200$ ) for individual and year effects (max. 1.05) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between year, within- and between-individual effects.**



**Figure S7.15: Precision analyses ( $n = 200$ ) for individual and cohort effects (max. 0.45) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between cohort, within- and between-individual effects.**



**Figure S7.16: Precision analyses ( $n = 200$ ) for individual and cohort effects (max. 1.35) among four batch sizes ( $n = 12$  to 48) using three sample allocation strategies: (1) aggregating samples per individual in the same batch (solid, red), (2) assigning samples randomly to batches (dashed, blue) or (3) slicing samples across batches with an angle that crosses two batches and a slicing width of a single batch (dotted, yellow). Raw data points from three separate simulations with mean imprecision per sample size are shown against among-batch variation, with 95% confidence intervals as shaded areas. Note, a precision value closer to zero means higher precision. Scales differ between cohort, within- and between-individual effects.**



## **Chapter 8: General discussion**

With the extensive evidence for senescence from natural populations of animals among many taxa (Nussey et al., 2013), we are currently acquiring an increasing understanding of the fundamental mechanisms of senescence and how environmental conditions are a source of the considerable variation in individual senescence patterns (Lemaitre et al., 2015; Cooper and Kruuk, 2018).

In this thesis, I have primarily focused on early-life conditions, when forces of natural selection are strongest (Hamilton, 1966; Williams, 1957; Medawar, 1952). I have used telomere length as a biomarker of senescence that reflects the physiological consequences of within-individual experiences by reflecting and individual's somatic state. In **Chapter 2** I found evidence for complex relationships between telomere length and age, with little within-individual consistency, where early-life telomere length predicted survival to adulthood and lifespan. In **Chapter 3**, I found that sex- and age-specific changes, dependent on the social conditions, contribute to variation in leukocyte cell composition which may affect the estimation of mean leukocyte telomere length. I then explored the interactive effects between the mean of and variability in early-life environmental conditions, specifically group size and food availability, which are reflected in early-life telomere lengths, in **Chapter 4**. In terms of transgenerational effects, in **Chapter 5** I found no evidence for sex-specific or within-parent age at conception effects on telomere length. Additionally, while additive genetic effects explained close to zero of phenotypic variation in telomere length, both cohort and year explained greater proportions of this phenotypic variance. In **Chapter 6**, I found no evidence that individuals anticipate their future somatic state through adaptive life-history strategies (i.e. internal PAR). However, I found some evidence that individuals anticipate the future adult environment, in support of the external PAR, but individual life-history was more dependent on the mean adult environment experienced. Finally, in **Chapter 7** I developed an approach to the analysis of samples in long-term studies, providing optimal statistical power and precision to detect year, within- and between-individual effects

while allowing convenient sequential analysis of collected samples, without confounding variables.

My thesis has contributed to our understanding of individual variation in telomere lengths and senescence patterns in wild mammals. It informs on how genetic and environmental factors, and adaptive life-history strategies, may shape individual variation in senescence patterns. Below, I discuss the key findings of my thesis and their implications for our understanding of telomeres and senescence, and highlight, where appropriate, future research objectives.

### **8.1 Telomere dynamics in European badgers**

Studies into telomere length in wild populations are mainly conducted in bird species (Boonekamp et al., 2014; Heidinger et al., 2012; Nettle et al., 2013b), but studies into wild mammals are emerging (Izzo et al., 2011; Lewin et al., 2015; Cram et al., 2017). However, few of those studies in mammals are longitudinal (Fairlie et al., 2016; Beirne et al., 2014), which is important to study within-individual changes such as senescence patterns. In **Chapter 2**, I investigated longitudinal telomere lengths among 24 cohorts in wild European badgers, and found support for complex relationships between telomere length and age, where early-life telomere length was linked to individual life-history.

The complex cross-sectional relationship between telomere length and age in **Chapter 2** was the result of both selective disappearance and within-individual changes. Within-individual consistency in telomere length was low, with both increases and decreases in telomere length with age that could not be explained by measurement error alone. The finding that telomere length forms such complex relationships with age contrasts studies conducted in humans (Baerlocher et al., 2003; Aubert and Lansdorp, 2008), but is in line with studies in other wild populations (Fairlie et al., 2016). For within-individual changes, a low repeatability in telomere length corroborates other qPCR studies across individual lifetimes

(Fairlie et al., 2016; Spurgin et al., 2017), but opposes telomere restriction fragment (TRF) studies conducted over shorter periods of time (Bauch et al., 2013; Boonekamp et al., 2014). Whether this discrepancy is the result of the method to estimate telomere length or a consequence of the period over which telomere length is estimated remains currently unclear. However, the high correlation between TRF and qPCR measurements in the Soay sheep indicates this cannot be attributed to methodology (Fairlie et al., 2016). I also found that decreases and increases in telomere length with age, and a resulting low within-individual consistency in telomere length, could not be attributed to measurement error as previously found in Seychelles warblers (*Acrocephalus sechellensis*; Spurgin et al. 2017). While in Seychelles warblers telomere length is estimated in erythrocytes, in mammals a competing mechanism for increases in telomere length with age exists. Since mammalian telomere length is estimated in leukocytes, changes in leukocyte cell composition with age may contribute to changes in longitudinal telomere lengths.

In **Chapter 3** I show sex- and age-specific changes in leukocyte cell composition in badgers dependent on group sizes. This means that social conditions have age- and sex-specific effects on innate and adaptive immunity, and these results are in line with studies in wild populations finding evidence for immunosenescence (Peters et al., 2019; Nussey et al., 2012; Cheynel et al., 2017). Additionally, these results indicate that changes in telomere length with age could be (partially) attributed to changes in leukocyte cell composition with age. This accords with a study in Soay sheep (*Ovis aries*), showing age- and sex-specific changes in leukocyte cell composition with age (Watson et al., 2017).

The additional evidence I provide for the complex relationship between telomere length and age and increases in telomere length with age, offers an exciting avenue for research into telomere elongation and its potential mechanisms (e.g. telomerase) in wild animals, and the consequences for life-history and senescence patterns. Furthermore, badgers had shorter early-life telomere length in winter compared to spring (**Chapter 4**), and

season explains variation in telomere length (**Chapter 5**). These seasonal effects indicate that torpor in badgers, or hibernation in other species, may be important for telomere lengthening. However, further research into the association between hibernation and telomere dynamics is required.

In **Chapter 2** I also found that early-life telomere length was linked to survival to adulthood and lifespan, providing additional evidence that in wild mammals (Fairlie et al., 2016; Cram et al., 2017), and in general (Wilbourn et al., 2018), telomere length functions as a biomarker of senescence. Finally, I found a lack of sex differences in telomere length in badgers. Even though badgers exhibit male-biased mortality (Bright Ross, unpublished data), this bias in mortality is relatively small compared to other species. Along with previous studies this indicates that sex differences in telomere length in wild mammals are in line with the degree of male-biased mortality. For example, Wytham badgers and meerkats show no or a small male-biased mortality and no sex differences in telomere length (Cram et al., 2017), whereas Soay sheep have a substantial male-biased mortality and show age-related sex differences in telomere length (Fairlie et al., 2016). While this contributes to the patterns in sex differences, the underlying mechanisms remain relatively unclear and require further investigation (Stindl, 2004; Gardner et al., 2014; Barrett and Richardson, 2011).

## **8.2 Early-life environmental conditions**

The early-life rather than later-life environmental conditions posit greater pressure on senescence patterns due to the forces of natural selection being strongest in early-life (Hamilton, 1966; Williams, 1957; Medawar, 1952). In **Chapter 2**, I found strong cohort effects on telomere length, indicating that early-life conditions are reflected in telomere length.

In **Chapter 5**, I found that variation in telomere length was explained by cohort and year effects, but with no evidence for parental or social group effects. These results indicate that variation in telomere length is a consequence of the birth year and capture year,

indicating the importance of environmental and ecological conditions. The individual variation in senescence patterns, for which telomere length functions as a biomarker, appears in badgers to be mainly determined by (early-life) environmental conditions.

Disentangling early-life environmental conditions into effects of weather conditions and group size was conducted in **Chapter 4**. I found that individuals experiencing higher levels of rainfall, with little variability, and higher temperatures had longer early-life telomere lengths. In badgers, weather conditions are linked to food availability, since badgers mainly feed on earthworms which are available in mild and moist conditions (Kruuk and Parish, 1981; Gerard, 1967; Newman et al., 2017). However, these effects of mean early-life environmental conditions interacted with the variability in weather conditions (Paaijmans et al., 2010; Bozinovic et al., 2011). Cubs experiencing warmer conditions had longer early-life telomere length. Yet, cubs experiencing lower mean daily temperatures benefitted from low variation in daily temperature, whereas with higher mean daily temperatures, variation in daily temperature had a beneficial effect on early-life RLTL. However, cubs experiencing higher mean daily rainfall benefitted from low variation in daily rainfall, whereas with lower mean daily rainfall, variation in daily rainfall had a beneficial effect on early-life telomere length. These results corroborate the finding that variability is linked to lower recruitment and survival probability in badgers when early-life environmental quality is high (Nouvellet et al., 2013). Variability in temperature (a proxy for food availability and thermal stress) can therefore result in maladaptation, particularly for traits such as senescence which are expressed later in life, and result in faster rates of senescence (Cotto and Ronce, 2014). In contrast, a temporal improvement in early-life environmental conditions through variability can provide individuals with additional resources and result in longer early-life telomere length (Erikstad et al., 1998). An adverse early-life environment may lead to a bet-hedging strategy, through investment in the soma at the expense of reproduction, modulating energy allocation (Morris et al., 2008; Wilbur and Rudolf, 2006). A

temporal increase in environmental quality, due to environmental variability, may thus change the balance in energy allocation trade-offs and life-history strategies (Erikstad et al., 1998; Reid et al., 2003; Weimerskirch et al., 2001). The contrasting effects of environmental variability provides opportunities for research, with expected increases in variability under climate change (IPCC, 2014), this may not only be important for habitat fragmentation and other ecological processes, but directly affect individual life-history. This requires further investigation in a variety of taxa with lifelong life-history data linked to mean early-life environmental conditions and variability in these conditions.

The quality of the early-life environment shapes individual life-histories (Reid et al., 2003; Nussey et al., 2007a) and explains variation in telomere length (Mizutani et al., 2013; McLennan et al., 2016; Watson et al., 2015; Wilbourn et al., 2017). In **Chapter 6** I found no evidence that individuals born in favourable conditions had longer lifespans than individual born in adverse conditions, in contrast to evidence for silver-spoon effects in other studies in wild populations (van de Pol et al., 2006; Cooper and Kruuk, 2018). However, besides a direct impact on individual life-history, adverse early-life conditions can constrain the development of the soma (Nettle et al., 2013a; Ricklefs and Wikelski, 2002). This can have an indirect effect on individual fitness through reduced energy allocation to somatic development, increase in damaging processes and reduced self-repair (Monaghan, 2014; Selman et al., 2012). Individuals in adverse early-life conditions, with detrimental effects on the developing soma, can anticipate their future somatic state and provide adaptive life-history strategies, known as the internal PAR (Nettle et al., 2013a). While studies in wild populations have found support for the internal PAR (Berghanel et al., 2016; Douhard et al., 2016) I found no evidence in **Chapter 6** that an individual can anticipate its own future somatic state (i.e. reflected in early-life telomere length) and thus the internal PAR. However, a large proportion of the variance in lifespan was still explained by cohort, which may be the consequence of lifespan strongly depending on whether an individual survives to adulthood.

Experienced early-life conditions can therefore influence life-history, but not through an adaptive response based on an individual's future somatic state. The combined effect of early-life adversity and consequences for the developing soma can still contribute to variation in senescence patterns, where further research among taxa is required to elucidate on the generality of such patterns.

Variability in environmental conditions on a longer scale can also impact senescence. I found in **Chapter 6** that individuals experiencing matching temperature conditions in early- and later-life exhibited longer lifespans, however not in terms of early-life telomere length or rainfall. These temperature results are in line with the external PAR, which predicts higher fitness when early- and later-life conditions match (Nettle et al., 2013a; Gluckman et al., 2005a; Gluckman et al., 2005b), but the telomere length and rainfall results do not support the external PAR. While in short-lived species the early-life and adult environment are more likely to match, leading to support for the external PAR (Dantzer et al., 2013; van den Heuvel et al., 2013), the autocorrelation of environmental quality is likely to be low in long-lived species and lead to rejection of the external PAR (Douhard et al., 2014; Lea et al., 2015; Hayward et al., 2013; Pigeon et al., 2017). The differences in the results based on the metric used for early-life environmental quality can be explained by the autocorrelation in these metrics and correlation between early- and later-life environmental quality. Metrics with a moderate to high autocorrelation, and high correlation between early- and later-life conditions (i.e. temperature) provide a higher likelihood for the external PAR compared to metrics with low autocorrelation and correlation between early- and later-life conditions (i.e. early-life telomere length/rainfall). The reason for this is that for the external PAR to be adaptive, greater environmental persistence is required (Fischer et al., 2011; Reed et al., 2010; Padilla and Adolph, 1996). Temporal fluctuations in the environment may thus reduce the benefits of the external PAR as an adaptive strategy (Wells, 2007; Rickard and Lummaa, 2007). Both short- and long-term variability, and associated quality, in environmental



conditions are predicted to increase under current climate change (IPCC, 2014) and further investigation of consequences of variability for individual variation in senescence patterns is required. However, further research to test the PAR hypotheses is required through the association between telomere length and reproductive success. Additionally, testing the relationship between telomere length and reproductive parameters, such as age at first breeding or early-life reproductive success in relation to lifespan, will elucidate on the PAR hypotheses and the selective pressures on telomere length.

Aside from anticipation of conditions experienced in adulthood based on the similarity with early-life environmental quality (**Chapter 6**), specific cues in early-life may lead to adaptive development. In **Chapter 4** I've shown that a higher number of cubs in the natal social group were associated with longer early-life telomere length. While this contrasts with some studies showing that higher levels of competition in early-life lead to accelerated telomere shortening (Boonekamp et al., 2014; Nettle et al., 2015) and shorter telomere length (Cram et al., 2017), this may reflect a plastic response to a social cue (Bretman et al., 2016; Kasumovic and Brooks, 2011). Badger cubs, particularly males, born in social groups with more cubs tend to have slower development, growth rates and reach sexual maturity later (Sugianto et al., 2019b) and this is thus associated with longer early-life telomere length. Smaller groups may therefore provide a competitive cue to cubs indicating to accelerate sexual development to utilise reproductive opportunities. Evidence for such plastic responses to competitive cues exist in fruitflies (*Drosophila melanogaster*; Bretman et al. 2016), and in wild populations (Brown and Brown, 2003; Lemaitre et al., 2011).

In **Chapter 3** I've also shown that the social environment throughout life leads to changes in immune profiles, with a stronger relative decrease in agranulocyte-to-granulocyte ratio for males in smaller groups. This accords with studies in wild animal populations that also show patterns of immunosenescence (Cheynel et al., 2017; Nussey et al., 2012; Peters et al., 2019). The social environment, and associated plastic responses and immune

challenges, may thus lead to variation in the rate of somatic development and as a consequence influence the patterns of senescence (Bretman et al., 2013; Leech et al., 2017). Further research in longitudinal studies can test the within-individual changes in absolute leukocyte numbers (e.g. through flow cytometry) and include these in the statistical models to fully understand the contribution of leukocyte cell composition to within-individual telomere dynamics.

Since conspecific competition in early-life showed strong effects on telomere length (Cram et al., 2017; Nettle et al., 2015; Boonekamp et al., 2014; Stier et al., 2015), and intra-sexual competition can impact body mass senescence as expected under sexual selection (Beirne et al., 2015), I investigated in **Chapter 4** whether intra-sexual competition in early adulthood was reflected in telomere length. A differential investment between reproduction and the soma can be expected between the sexes, due to divergent life-history strategies (Bonduriansky et al., 2008; Singh and Punzalan, 2018). While in polygynandrous species a male-biased mortality, because males benefit from many matings they might experience greater intra-sexual competition (Bonduriansky et al., 2008; Lemaitre et al., 2014; Beirne et al., 2015). However, I found no evidence that intra-sexual competition was reflected in telomere length. Interestingly, in another badger population an effect of intra-sexual competition on body mass senescence was found (Beirne et al., 2015). In Wytham badgers there is strong reproductive skew among males, where badgers with a higher body condition index attain higher reproductive success (Dugdale et al., 2008), as a result of a competitive advantage. However, high levels of extra-group paternity (Dugdale et al., 2007; Annavi et al., 2014b), promiscuity among males and repeated mounting behaviour (Dugdale et al., 2011a) may mask paternity. Additionally, female badgers exhibit various postcopulatory mechanisms (i.e. delayed implantation, superfetation, superfecundation; Yamaguchi et al., 2006), where this cryptic female choice may mask paternity, reduce male-male aggression

and thus reduce intra-sexual competition. Further studies in populations with sex-biased mortality rates are required to determine whether such effects are reflected in telomeres.

### **8.3 Transgenerational effects**

The evolutionary potential of trait such as telomere length, according to the breeder's equation, depends on the heritable component and the strength of natural selection (Lynch and Walsh, 1998). In **Chapter 5**, I found that the heritability of early-life telomere length and telomere length across and individual's lifetime was close to zero. This contrasts with other studies that found diverging rates of heritability estimates, but were mainly conducted with parent-offspring regression which cannot separate 'shared environment' effects between offspring and their parents (Voillemot et al., 2012; Reichert et al., 2015; Olsson et al., 2011; Horn et al., 2011). The 'animal model' can cope with these effects. Using the animal model in wild vertebrate populations, Becker et al. (2015) found no heritability of telomere length in white-throated dippers, whereas in Great reed warblers relatively high heritability of telomere length was found (Asghar et al., 2015a). However, these studies could not separate permanent environment effects and did not provide a power analysis. While we therefore still have a limited understanding of heritability of telomere length in wild populations, we know even less of the heritability of telomere shortening. Further research with greater sample sizes, power analyses, looking at the heritability of telomere length and telomere shortening are required.

In **Chapter 5**, I also found that neither paternal nor maternal age at conception was associated with offspring telomere length. Additionally, I found no evidence of sex-specific or within-parent age at conception effects. While a relationship between paternal age at conception and offspring telomere length has been found in humans (Njajou et al., 2007; Hjelmborg et al., 2015; Kimura et al., 2008) and chimpanzees (Eisenberg et al., 2017), another recent study found no support for such effects in Soay sheep (Froy et al., 2017). While the

paternal age at conception effect could have been through increased telomerase activity in the male germline to counteract telomere loss, or the survival of germ stem cells with longer telomeres that subsequently predominate the germ stem cell pool at older ages (Kimura et al., 2008), I found no support for such effects. Bouwhuis et al. (2018) suggested that parental age at conception effects depend on the mating system with positive effects in species with year-round sperm production, whereas species with seasonal peaks or monogamous species show a negative association with telomere length. Badgers have a polygynadrous mating system and should exhibit high levels of sperm production and competition, but do not show any parental age at conception effects. While initial studies in humans showed strong paternal age at conception effects (Njajou et al., 2007; Hjelmborg et al., 2015; Kimura et al., 2008), such transgenerational effects in badgers and other wild mammals (Froy et al., 2017), and other taxa (Heidinger et al., 2016; Olsson et al., 2011; Asghar et al., 2015a; McLennan et al., 2018) may not be straightforward and require further investigation.

#### **8.4 Analysis of long-term studies**

All of the above results require separation of confounding variables from variables of interest, which is often problematic in long-term studies due to cross-classified data structures. In **Chapter 7**, I investigated the current approaches to the analysis of samples in long-term studies and the potential issues with these approaches. While long-term studies provide an important tool for ecological and evolutionary studies (Clutton-Brock and Sheldon, 2010), the analysis of such comprehensive datasets can be complex due to hierarchically structured data and confounding effects. The often naturally cross-classified data structures in long-term studies can be overcome with mixed models (Schielzeth and Nakagawa, 2013; Gelman and Hill, 2006; Bolker et al., 2009) or when experimental design leads to nested data. However, separating variation in sources of interest from confounding effects is difficult with current approaches. I therefore developed an approach termed slicing

that analyses samples collected at different times together in a batch and cluster. The attributes of samples are therefore not fully confounded with the attributes of batch, allowing separation of variation of interest from potential confounds. Additionally, slicing allows convenient sequential analysis of samples while maintaining statistical independence. This novel slicing approach might pose an outcome in the molecular analyses of samples in long-term studies, but more generally the approach to structuring samples for analysis and the statistical considerations should be considered and reported in such studies. The slicing chapter was developed after conducting my laboratory work, so was not implemented in this thesis, but represents a novel method that will improve our understanding of diversity in telomere dynamics in the future.

### **8.5 Concluding remarks**

The conditions that influence individual variation in senescence patterns in natural populations of mammals is still progressing. My thesis improves understanding of factors linked to variation in the onset and rate of senescence, using telomeres as a biomarker of senescence. I have provided additional evidence for links between individual life-history and early-life telomere length in wild mammals. My research highlights the importance of early-life environmental conditions, in contrast to additive genetic and transgenerational effects, and the potential for adaptive life-history strategies in anticipation of an individual's future environment. I show that both the mean of and variability in the early-life environment are reflected in early-life telomere length, indicating a role for environmental conditions during the developmental phase. In badgers we show that weather conditions, as a proxy for food availability, and plastic responses to natal group size cues explain variation in early-life telomere length. While it is apparent that longitudinal studies among taxa are required to explain the individual variation within and between species, such studies require an accurate approach to analysis for which I consider the novel 'slicing' approach.

Even though the early-life environment and adaptive strategies are important for telomere dynamics and senescence patterns in badgers, much remains to be learned about telomere dynamics and individual variation in senescence patterns. Future research in diverse taxa, with assorted mating systems and population dynamics will inform on why some species show paternal age at conception effects or support for the internal and external PAR where other species do not. Such varied information will elucidate on the evolved patterns of senescence and why the considerable individual variation in senescence patterns exists.

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