Measuring and Understanding Senescence in a wild population

Thomas James Brown

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School of Biological Sciences

University of East Anglia

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Abstract

Senescence – the decline in organismal function and performance – frequently occurs in many taxa. However, within- and between- populations of the same species there can be considerable variation in the onset and rate of senescence. Understanding the causes and fitness consequences of differential senescence is a key objective in the study of life-history evolution and gerontology. Wild populations are uniquely suited for exploring the role of complex genetic, environmental and social contributions to senescence patterns. However, accurately measuring the severity of senescence at the individual-level has presented a long-standing challenge. In this thesis, I explore the utility of several candidate intrinsic markers of senescence in a closely monitored wild population of Seychelles warblers (*Acrocephalus sechellensis*). Firstly, I reveal complex relationships between haematocrit, age a survival, which suggest haematocrit could be used as a marker of early-life stress. Secondly, I show that telomere dynamics (i.e. the increase and decrease in telomere length within-individuals) reflect life-history costs in females, such as reproductive effort and malarial infections, and subsequently impacts future mortality risk. Thirdly, I find evidence of a silver-spoon effect, whereby individuals in better condition (mass adjusted size) as juveniles have higher survival probabilities throughout adult-life but does not affect patterns of survival- or reproductive senescence. Lastly, I develop a ribosomal DNA-methylation clock for predicting age and survival, with substantially improved accuracy over previously used markers; telomere length and condition. With the combined use of multiple intrinsic markers, this study reveals the dynamic and varied ways in which fitness-related traits change with age in a single species. Based on these findings, I discuss the relative strengths and weaknesses of different intrinsic markers for improving our understanding of ageing, senescence and life-history in wild populations.

Access Condition and Agreement

Contents

Chapter Contributions

At the time of submission, two of the four data chapters in this thesis have been published in peer-reviewed journals. The third data chapter is also being revised for re-submission, following reviewer's comments. For all manuscripts I am the lead author and responsible for the largest contribution. Below, I provide co-author information and detail specific contributions I have made to each data chapter.

- Chapter 2: Brown, T.J., Hammers, M., Taylor, M., Dugdale, H.L., Komdeur, J. and Richardson, D.S. (2021). Hematocrit, age, and survival in a wild vertebrate population. Ecology and Evolution, 11(1), pp.214-226. https://doi.org/10.1002/ece3.7015
  I compiled and analysed the data, and drafted the manuscript (80%)

- Chapter 3: Brown, T. J., Spurgin, L. G., Dugdale, H. L., Komdeur, J., Burke, T., & Richardson, D. S. (2021). Causes and consequences of telomere lengthening in a wild vertebrate population. Molecular Ecology, 00, 1– 13. https://doi.org/10.1111/mec.16059
  I compiled and analysed the data, and drafted the manuscript (80%)

- Chapter 4: Brown, T.J., Dugdale, H.L., Hammers, M., Komdeur, J. and Richardson, D.S. (2021). Seychelles warblers with silver spoons: juvenile condition is a lifelong predictor of annual survival, but not annual reproduction or senescence (In review: preprint doi: 10.22541/au.163664637.70605525/v1
  I compiled and analysed the data, and drafted the manuscript (80%)

- Chapter 5: Brown, T.J., Taylor, M., Dugdale, H.L., Burke, T., Komdeur, J. and Richardson, D.S. (2022). Epigenetic markers of chronological age and survival in a wild passerine (In prep).
  I performed the lab work (sample prep etc.), compiled and analysed the data, and drafted the manuscript (80%)

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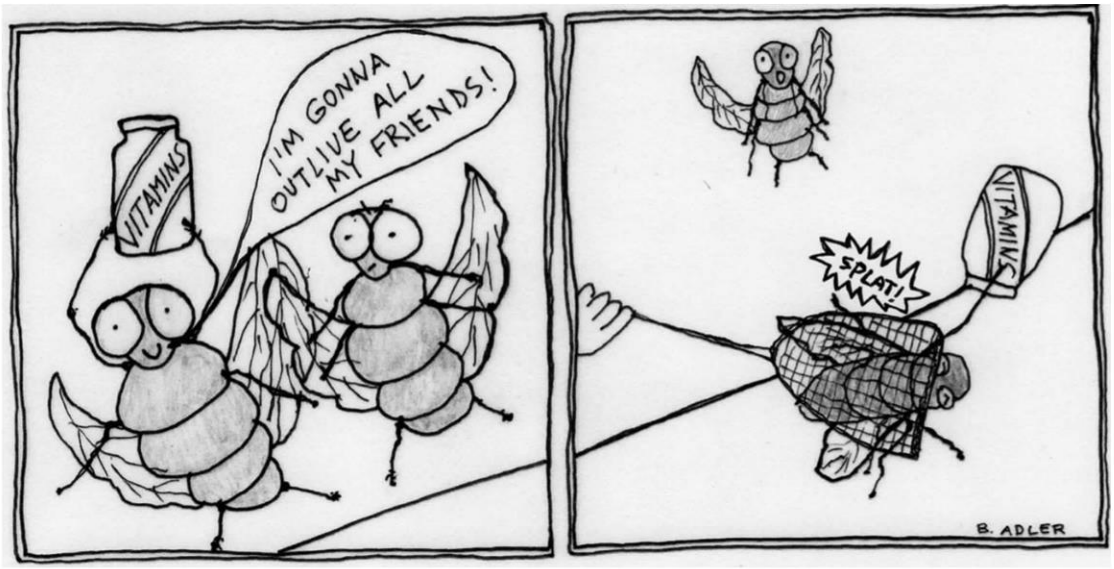
Chapter 1
Introduction



Investing in somatic maintenance is unlikely to pay off when the risk of extrinsic mortality is high – Adler and Russell 2014

1.1. General introduction

Senescence – defined as the biological deterioration of an organism with advancing age – results in age-related declines in performance and fitness (e.g. survival and reproduction) in late-life (Monaghan *et al.*, 2008a; Ricklefs, 2008). The proximate cause of senescence is an accumulation of damage to the soma via a suite of interacting molecular, cellular and physiological pathways; referred to as the 'hallmarks of ageing' (Kirkwood, 2005, 2008; López-Otín *et al.*, 2013). Senescence is common across animal taxa, and observed in laboratory, captive and wild environments (Nussey *et al.*, 2008). However, the pattern of senescence (i.e., onset and rate) differs among species; some exhibit rapid senescence while others seemingly avoid noticeable fitness declines (Jones *et al.*, 2013). Why senescence patterns have evolved to be more severe in some species than others has become a longstanding question in evolutionary biology (Williams *et al.*, 2006).

The rate and onset of senescence can also be highly variable among individuals within populations (Williams *et al.*, 2006; Charmantier, Brommer and Nussey, 2013; Nussey *et al.*, 2013). Thus, individuals of the same chronological age may exhibit different 'biological' ages; deviations from the population mean health for a given chronological age (Jylhävä, Pedersen and Hägg, 2017). Understanding what factors modulate ageing to drive this variation, and why, is fundamental to our understanding of life-history evolution, and has important applications in medicine. For example, increases in average human lifespan, but not "healthspan" (the period of disease-free life), over recent decades has created an urgent need for medical interventions to delay or alleviate the senescent phenotype (Kirkwood, 2008; Guarente, 2014).

To identify factors that explain variation in senescence, and/or test interventions that could lessen senescence, we first need to be able to measure biological age. Given the complex and variable nature of senescence, the challenge is to identify markers that reflect processes common to the multiple ageing mechanisms, or at least those which contribute most to the senescent phenotype (Jylhävä, Pedersen and Hägg, 2017). A universal biological age marker – that accurately predicts the future onset and severity of age-related declines in performance, independently of chronological age, across taxa – has proven elusive.

1.1.1. Theories of ageing

Senescence results in an age-related decline in Darwinian fitness, posing the question why it has not been eliminated by natural selection. Three mutually inclusive hypotheses have been proposed to explain and unify the diversity of ageing patterns across taxa. Extrinsic mortality (EM; due to e.g. disease, predation etc.) reduces the likelihood of surviving to old age, meaning the strength of selection declines with age (Hamilton, 1966). Therefore, mutations with deleterious effects in late-life are, to some extent, hidden from selection – hence the term "selection shadow" – and thus can accumulate in populations; the Mutation Accumulation hypothesis (Medawar, 1952). Williams

(1957) further reasoned that alleles with late-life deleterious effects will still be positively selected if those same alleles convey fitness gains (i.e. increased reproduction) in early-life - thus increasing lifetime fitness at the expense of late-life performance; the Antagonistic Pleiotropy (AP) hypothesis. A similar trade-off is predicted by Kirkwood's (1977) Disposable Soma (DS) hypothesis, which focuses on the allocation of limited resources, namely energy, to physiological mechanisms of somatic maintenance/repair versus investments in growth and reproduction. Natural selection optimizes the balance of resource allocation to these competing traits in a way that maximizes lifetime fitness, rather than longevity or late-life performance. Therefore, the constant "wear-and-tear" of soma accumulates, leading to senescence.

1.1.2. Extrinsic mortality

The overarching basis of all three hypotheses is that senescence evolves under the selection shadow of EM. Therefore, ageing theory predicts a positive association between EM rates and senescence, mediated by higher mutational load (MA) and/or greater early-life investments (AP and DS). Cross-species comparisons of survival and fecundity trajectories generally support this prediction; species with high EM senescence faster and produce a higher proportion of total offspring in early-life (Jones *et al.*, 2008, 2013). Likewise, species with adaptations/life-histories associated with lower EM rates (e.g. flight, bigger brains, social structures) have longer lifespans, and relatively constant fecundity across their lifetime (Ricklefs, 1998; Bourke, 2007; Healy *et al.*, 2014). A notable caveat to this association is taxa with high EM rates in early-life that diminish with age, accompanied by an increase in fecundity. Such is the case for species with protracted growth (e.g. turtles, large fish), which typically escape EM pressures (i.e. predation) and are more fecund with increasing size (Vaupel *et al.*, 2004; Jones *et al.*, 2013).

The within-species effect of EM on senescence is less clear-cut and likely depends on the prevailing cause of EM (Bronikowski and Promislow, 2005; Gaillard and Lemaître, 2017). In nature, EM is rarely independent of individual condition, since survivors will be those that are better able to resist agents of EM. For example, contrary to expectations, when condition dependence plays a role, higher rates of EM can induce selection for longer lifespans in experimental lines of nematodes (*Caenorhabditis elegans*; Chen and Maklakov, 2012). Similarly, Trinidadian guppy (*Poecilia reticulate*) populations subject to higher EM have higher reproductive investments (as expected by ageing theory), but without inducing faster actuarial senescence (Reznick *et al.*, 2004). These observations have led to the proposed hypotheses of positive pleiotropy – positive genetic correlations between early- and late-life performance – and modified-MA – mutations with major deleterious effects in late-life (i.e. classic MA) being eliminated under high condition-dependent EM due to minor deleterious effects in early-life (Maklakov, Rowe and Friberg, 2015).

1.1.3. Life-history trade-offs and constraints

Both AP and DS predict a trade-off between somatic maintenance and growth/reproduction that maximises fitness, with senescence as a secondary outcome. Life-history trade-offs (LHT) occur when gains in one fitness component (e.g., reproduction) are associated with costs to another fitness component (e.g. survival). Cost-benefits can be realised at different stages in an individual's lifetime, such that the initial benefits of fast growth and/or earlier reproduction have delayed costs for late-life performance. Since the optimal life-history (for maximal fitness) depends on an organism's ecological context (such as EM rate), AP and DP are collectively referred to as life-history theories of ageing (Partridge and Barton, 1996). There is strong comparative support for early-late LHTs across taxa; early-life performance (fecundity and reproductive onset) accounts for *ca* two-thirds of the variation in age at onset of survival senescence and longevity (Peron *et al.*, 2010; Healy *et al.*, 2019). Therefore, species are considered to lie on a fast-slow continuum, characterised by early-reproduction and short-lifespans at the fast-end and delayed-reproduction and long-lifespans at the slow-end (Jones *et al.*, 2013).

There is also strong support for the early-late LHTs modulating senescence within species. Lemaître's *et al.* (2015) review of 26 field studies (that explicitly tested for early-late LHTs in performance within wild populations) showed that 21 (*ca* three-quarters) found evidence for trade-offs; individuals who invest earlier, or more heavily, in reproduction/growth have an earlier onset and/or faster rate of senescence (e.g. Nussey *et al.*, 2006; Boonekamp *et al.*, 2014). Differing senescence trajectories between males and females of the same species can also be explained by differing LHT optima (Bonduriansky *et al.*, 2008). In males, sexual selection is greater (owing to females being the limiting sex) leading to increased conflict and sexually-selected traits (Clutton-Brock, 2007), and ultimately to higher EM (relative to females). Therefore, the observation that males are generally shorter-lived and/or senescence faster than females (at least in mammals; Marais *et al.*, 2018) can be attributed to faster life-histories, i.e., males allocating greater resources into the growth/maintenance of sexually-selected traits over somatic-maintenance, relative to females (Carranza *et al.*, 2004; Balbontín *et al.*, 2011).

LHTs have become a broadly accepted paradigm in ageing research (for a recent review see Gaillard and Lemaître, 2020). However, LHTs alone are unable to explain the diversity of taxonomic- and sex-specific ageing; there are numerous ecological and evolutionary constraints within which LHTs operate (e.g. Marais *et al.*, 2018; Healy *et al.*, 2019). Within-species, there are also important conditions and caveats to the role of LHTs in ageing (discussed in detail in Cohen *et al.*, 2020). For example, positive pleiotropy suggests some genes/mechanisms associated with increased early-life investments are not necessarily costly, or may even be beneficial to late-life performance (Maklakov, Rowe and Friberg, 2015). That said, the majority of observed positive pleiotropy

originates from model organisms in benign laboratory-environments, which could alleviate the costs of early-life investments on late-life performance. In wild populations, it is evident that the costs of reproduction on survival may only occur when/where resources are limiting (Tavecchia *et al.*, 2005; Garnier *et al.*, 2016). Similarly, there is little evidence of reproductive costs on the lifespan of vertebrate populations in captivity – where resources are non-limiting (Ricklefs and Cadena, 2007). Therefore, environmental variation can modulate senescence trajectories by strengthening or weakening early-late LHTs.

As well as environmental variation, variation in resource acquisition is expected to exist in in wild populations due to, for example, differences in individual quality (also see silver-spoon effects in Chapter 4). That some individuals are better able to acquire resources (relative to others) suggests those same individuals will perform better in both early- and late-life despite the existence to trade-offs (van Noordwijk and de Jong, 1986; Descamps *et al.*, 2016). For example, in female southern elephant seals (*Mirounga leonina*) early- and late-life performance is positively correlated, supporting positive pleiotropy (assuming a genetic basis) and individual-quality hypotheses (Oosthuizen *et al.*, 2021). Another interesting case study is provided by facultative cooperative breeding systems, whereby the acquisition of helpers can both improve reproductive success of primary breeders (i.e., by acquiring more resources) without incurring costs to primary breeders late-life performance (Berger *et al.*, 2018; van Boheemen *et al.*, 2019; Hammers *et al.*, 2021).

Many of the molecular and cellular mechanisms of senescence (i.e., the Hallmarks of ageing; López-Otín *et al.*, 2013) fit within the LHT-framework of ageing. A classic example is the role of insulin-like signalling pathways in promoting cellular growth and reproduction (in high nutrient environments), and cellular protection/maintenance (in nutrient-limited environments). Broadly speaking, reduced activity of insulin-like signalling (e.g. via dietary restriction) extends lifespan and improves late-life health, often at a cost to growth and fecundity (Tatar, Bartke and Antebi, 2003; Fontana, Partridge and Longo, 2010). Similarly, it has recently been argued that increases in cancer risk with age may reflect resource-allocation trade-offs between growth/reproduction and immunocompetence i.e., increased investments in growth/reproduction increases cancer risk via faster senescence of the immune system (immunosenescence) that would otherwise eliminate cancer cells (Boddy *et al.*, 2015; Lemaître *et al.*, 2020). Therefore, insulin-like signalling appears to be an influential mediator of early-late LHTs (for reviews see Dantzer and Swanson, 2012; Regan *et al.*, 2020).

In contrast to insulin-like signalling, other ageing mechanisms act more as constraints on late-life performance, either because they are not subject to obvious modulation via resource allocation, or because they are linked to essential functions in early-life (discussed in Cohen *et al.*, 2020). For example, telomere length attrition – a contributor to somatic deterioration – occurs with age despite the existence of restorative mechanisms (see Chapter 3 for full discussion). Why these

mechanisms are inactive (generally) in adult-life could be due to telomere attrition and linked pathways (e.g. cellular senescence and inflammation) being involved in cancer prevention (Shay and Wright, 2011; Schosserer, Grillari and Breitenbach, 2017). Similarly, strong immune responses are associated with increased tissue inflammation in late-life, yet are crucial for preventing infection-mortality in early-life (Metcalf, Roth and Graham, 2020). Therefore, mechanisms may have harmful effects in later-life (i.e. under the selection shadow) as a consequence of being optimized for early-life functions; the hyperfunction hypothesis (Lind *et al.*, 2019; Maklakov and Chapman, 2019)

1.1.4. Genetic origins

AP assumes a genetic basis of early-late LHTs. Since Williams' 1957 article, there have been numerous discoveries of gene mutations, knock-outs and knock-ins that alter longevity in laboratory organisms (Partridge and Barton, 1994; Selman *et al.*, 2008; Maklakov *et al.*, 2017), which typically exert their effects by downregulating metabolic pathways and activating targets involved in cell maintenance. For example, reduced activity in Insulin-Like Growth Factor (IGF)-1 receptor can extend the lifespan of yeast, nematodes, insects and mammals (Tatar, 2003; Guarente, 2014). Life-extending gene modifications are also often (but not always) detrimental to early-life fitness (Table 1 in Nussey *et al.*, 2013); thus supporting AP and DS.

However, the extent to which such gene-variants determine senescence in non-laboratory organisms is unclear (Leroi *et al.*, 2005; Nussey *et al.*, 2013; Yadav *et al.*, 2015). Laboratory organisms tend to be more genetically uniform than wild populations, which may exacerbate the phenotypic expression of single life-extending genes (Ricklefs, 2008). Secondly, the life-extending and AP-effects of such genes are likely to be resource-dependent (as discussed above), and thus have varying phenotypic outcomes in wild environments (e.g. Kawasaki *et al.*, 2008; Nussey *et al.*, 2013). Indeed, such life-extending gene variants are likely to be eliminated in wild settings due to AP-effects, i.e., lower performance in early-life relative to wild-type (Jenkins, McColl and Lithgow, 2004; Maklakov *et al.*, 2017b). Therefore, single-gene models, though insightful, represent an over-simplification of senescence in wild organisms.

Quantitative genetic methods (e.g., through the use of animal models etc.) applied to datasets from wild populations indicate that some phenotypic variation in senescence does have a genetic basis (reviewed in Charmantier *et al*. 2013). Notably, in mute swans (*Cygnus olor*), and red deer (*Cervus elaphus*), genetic negative co-variance exists between early- and late-life reproductive performance; thus supporting AP (Charmantier *et al.*, 2006; Nussey *et al.*, 2008). However, unlike single-gene models (see above), it is difficult to demonstrate causality with quantitative genetic approaches, i.e., genetic covariation may be unrelated to the causal mechanisms of LHTs and/or senescence (Ricklefs, 2008). According to AP and DS, senescence is an outcome of optimized LHT,

which suggests gene-variants with beneficial AP-effects should quickly go to fixation (Partridge and Barton, 1993). Therefore, selection in wild populations should erode the causative genetic components of differential senescence (Leroi *et al.*, 2005).

1.1.5. Environment and ageing plasticity

It has long been acknowledged that a large component of individual variation in senescence has non-genetic origins (Cournil and Kirkwood, 2001). For example, human monozygotic twins can exhibit differing senescence trajectories (Steves, Spector and Jackson, 2012). In the absence of genetic and environmental variation, between-individual differences in senescence trajectories may still occur due to minor stochastic events (e.g., DNA-replication error, epigenetic alterations) that accumulate within the soma (e.g., Vogt *et al.*, 2008). However, populations in real-world settings are subject to high levels of both environmental and genetic variation, meaning there is great potential for environmental factors (and gene-by-environment interactions) to modulate senescence patterns.

In wild populations, environmental factors can have a direct impact on late-life performance. For example, older individuals may only exhibit performance declines (or exhibit more severe performance declines) in adverse environments (Nussey *et al.*, 2013 and references therein). However, environmental factors have arguably the greatest influence on senescence during early-life. Life-history theories of ageing predict early-life to be a critical period in which available resources are partitioned between growth, first reproduction, and somatic maintenance (Lemaitre et al., 2015). Additionally, the somatic damage incurred during early-life (i.e., during development) is thought to be equal to or greater than the damage accumulated during adult-life; the High Initial Damage Load (HIDL) hypothesis (Gavrilov and Gavrilova, 2004). Therefore, small differences in early-life environment are predicted to have exaggerated effects on senescence.

There is strong support for environmental factors in early-life influencing senescence in wild populations (discussed in detail in the introduction of Chapter 4). Briefly, more favourable environments (commonly measured in terms of food availability, population density, weather etc.) generally benefit both adult and late-life performance; termed “silver-spoon” effects (Lindström, 1999; Metcalfe and Monaghan, 2001; Daniel H. Nussey *et al.*, 2007; Cooper and Kruuk, 2018). Such observations are also consistent with other constraint-based theories of senescence, i.e., individual-quality and positive pleiotropy.

Early-life environments could bring about adaptive shifts in adult phenotype and life-history, with differing outcomes for senescence, e.g., internal Predictive Adaptive Responses (PARs), ‘thrifty’ phenotype, resource-reallocation hypothesis (Shanley and Kirkwood, 2000; Hales and Barker, 2001; Monaghan, 2008; Bateson, Gluckman and Hanson, 2014). Following DS principles, these hypotheses predict that the optimal early-late LHT is modified by early-life environment. For example, a silver-

spoon upbringing may enable maximum resource allocation to growth and reproduction in early-life, to the detriment of late-life performance, whereas harsher environments restrict and/or delay early-life investments to the benefit of late-life performance (Hunt *et al.*, 2004; Hooper *et al.*, 2017; Spagopoulou *et al.*, 2020).

Nevertheless, environmental factors throughout adult-life are important and may interact with the effects of early-life environments to produce varying senescence outcomes (Douhard *et al.*, 2014; Berger *et al.*, 2015, 2018). Indeed, the outcome of hypothetical-PARs (i.e. whether they have positive or negative effects on adult performance) depends on the strength of correlation between early-life- and adult-life environment (Gluckman, Hanson and Spencer, 2005). Regardless of early-life environmental effects, environmental factors during reproduction still influence reproductive costs to somatic maintenance (e.g. Reed *et al.*, 2008; Harding *et al.*, 2011). Furthermore, in some taxa, extreme adverse environments during adulthood can trigger discrete periods of dormancy (e.g. torpor) in which reproductive investments cease in favour of somatic maintenance and life-span extension (Flatt *et al.*, 2013; Wu and Storey, 2016).

The observed effects of the environment on senescence imply that underlying mechanisms of ageing are sensitive to environmental exposures, and disproportionately so in early-life. One of the most robust and widely studied examples is dietary-restricted insulin-like-signalling. In model organisms raised under dietary restriction, the downstream effects on insulin-like signalling result in marked decreases in senescence and extended lifespans (e.g. mice live 30-50% longer) compared to populations of the same organism raised on *ad libitum* diets (Fontana, Partridge and Longo, 2010). The late-life benefits of dietary restriction have been explained in terms of DS or, more specifically, the resource-reallocation hypothesis; shifting resources from growth/reproduction when food is scarce in favour of somatic maintenance and the preservation of reproductive value for when food becomes non-limiting (Shanley and Kirkwood, 2000). This suggests that some ageing pathways may have built-in plasticity that adjusts individual life-histories to environmental conditions (Regan *et al.*, 2020).

Support for the resource-reallocation hypothesis is mixed, since the expected fecundity costs and increased resilience of model organisms subject to dietary restriction are inconsistent and often conditional (Burger *et al.*, 2007; Auer, 2010; Adler *et al.*, 2013; McCracken *et al.*, 2020). Furthermore, it is unclear whether dietary restriction effects are applicable or relevant to senescence in wild populations. Adler and Bonduriansky, 2014, argue that the wider downstream effects of dietary restriction (including compromised immunity) will likely increase early-life mortality in wild settings; thus, any potential benefit to late-life performance will not be realised. Furthermore, early-life stress and compensatory growth (following delayed development) are known to accelerate multiple interconnected ageing pathways, such as telomere attrition,

inflammation and immunosenescence (Metcalfe and Monaghan, 2001; Von Zglinicki, 2002; Nettle *et al.*, 2017; Angelier *et al.*, 2018). This is perhaps why positive associations between early-life environment and late-life performance (i.e., silver-spoon effects) seem to be more prevalent in wild populations (Cooper and Kruuk, 2018, but see Spagopoulou *et al.*, 2020).

1.2. Measuring senescence in the wild

The ageing literature indicates that patterns and drivers of senescence observed in the laboratory model organisms cannot necessarily be generalised to wild populations. Non-model organisms in real-world settings provide a unique (but largely untapped) resource for exploring complex genetic, environmental and social effects on senescence (Gaillard and Lemaître, 2020). Improved understanding of ageing in wild taxa is also likely to have greater application (i.e., for anti-ageing interventions) to medicinal, veterinary and conservation fields.

1.2.1. Study design

The study of ageing in wild populations does, however, pose many changes – not least the need to accurately chronologically age individuals (Jarman et al., 2015). Few species possess external features from which chronological age can be determined reliably (e.g. tooth wear in deer; Pérez-Barbería et al., 2014) and molecular tools that can estimate age from DNA samples have only started to emerge relatively recently (De Paoli-Iseppi, Deagle, *et al.*, 2017). The solution has been to uniquely mark young individuals (with tags, leg rings etc.) – so that the same individual caught early in life can be re-sighted/re-captured and aged at later dates. However, it is often extremely difficult, costly, or impractical to continuously monitor large numbers of individuals as they age in the wild.

Importantly, many field studies are cross-sectional in design, comparing the performance of different individuals in different age cohorts (Nussey *et al.*, 2013). However, cross-sectional studies are often confounded by selective disappearance issues, whereby the likelihood of reaching old age varies between phenotypes. For example, in roe deer (*Capreolus capreolus*) body mass declines within-individuals with age, reflecting a senescence of body condition. However, since individuals with low body mass have a greater mortality risk, and thus "disappear" at younger ages, the population-mean body mass increases with age (Nussey *et al.*, 2008). Thus, cross-sectional differences in traits do not necessarily reflect the within-individual senescence of traits (Reid *et al.*, 2003; Nussey *et al.*, 2008).

Longitudinal studies, whereby marked individuals are monitored throughout their lives, overcome such selective disappearance issues. The value of longitudinal studies of wild populations in senescence research has been emphasized over the past decade (Nussey *et al.*, 2008; Nussey *et al.*, 2013). Such studies require populations with individuals than can be readily observed (e.g., for

reproductive success data) and repeatedly captured (to take intrinsic measures of physiological senescence, see below). Several decades of continued monitoring may be required to accumulate large enough datasets of complete lifetime data (i.e. from birth to death), especially for long-lived species, or systems where relatively few individuals reach senescent age; for example, due to high rates of early-life EM. Furthermore, in natural wild populations corpses are rarely recovered from study areas. Consequently, field studies are often forced to assume individual death date (or age-at-death) from a lack of continued detections/recaptures, which in some systems can be confounded by dispersal outside of the study area.

1.2.2. Demographic measures

There are multiple ways in which senescence can be measured in wild populations, each with its advantages and limitations. The simplest and easiest metrics for obtaining population-level estimates of senescence are derived from individual age-at-death (assuming age-at-death can be measured, see above). In comparative studies, maximum life-span or life-span distributions (e.g. average longevity or 90% longevity) are commonly used as proxies for "*rate-of-ageing*" (e.g. Møller, 2007). However, lifespan metrics by themselves are not a measure of senescence since they only capture an end-point, not the "shape" (i.e. onset and rate) of performance declines with age (Monaghan *et al.*, 2008b). Shape can be incorporated lifespan metrics with the use of time scales (i.e. time-to-death) to calculate survival curves and/or mortality risk relative to age (Ronget and Gaillard, 2020).

Within mixed-model frameworks, survival curves can be assessed in relation to other factors, while controlling for age, to determine their effect on actuarial senescence. For example, a comparative-analysis of Soay sheep (*Ovis aries*) reveals that actuarial senescence is broadly similar between sexes, despite males having considerably higher mortality risk overall (irrespective of age) and shorter average lifespan compared to females (Colchero and Clark, 2012). However, neither age-at-death nor survival curves are informative of senescence at the intrinsic- or individual-level. In wild populations, it cannot be assumed that an earlier/later age-at-death corresponds to accelerated/delayed physiological senescence, or higher/lower biological age (Monaghan *et al.*, 2008b; Ricklefs, 2008). Additionally, age-at-death provides only one datum per individual (i.e., an individual can only die once), meaning within-individual changes in performance cannot be measured.

For iteroparous species (i.e., where individuals have multiple reproductive events throughout their lives) metrics of reproductive functionality (e.g. ejaculate quality) and reproductive success (e.g., offspring quantity/quality) can be used as metrics of within-individual senescence. In wild taxa, reproductive senescence is widespread in both males and females, which can reflect both physiological deterioration (i.e., of reproductive systems) and/or a diminishing ability to

acquire/allocate resources to reproduction or sexual-competitiveness, e.g., the maintenance of sexually-selected traits in males (for reviews see Nussey *et al.*, 2013; Lemaître and Gaillard, 2017). Measuring age-specific reproduction can also reveal LHTs between early- and late-life performance i.e. negative associations between early-life reproductive investments and late-life reproductive performance (Bouwhuis *et al.*, 2010) and survival (Hammers *et al.*, 2013; Boonekamp *et al.*, 2014).

There are, however, many factors that can cause a disassociation between age-specific reproductive success (e.g., offspring quantity/quality) and physiological senescence. Increased resource-acquisition with age (via gains in experience, helpers etc.) can alleviate both reproductive- and physiological senescence (e.g. DuVal, 2012; Hammers *et al.*, 2019). Similarly, increases in reproductive effort with age – termed terminal investment strategies (Clutton-Brock, 1984) – can maintain and/or increase age-specific reproductive performance, in spite of – or contributing to – physiological senescence (Massot *et al.*, 2011; Froy *et al.*, 2013). Furthermore, since reproduction is an outcome of male-female interactions, it may be difficult to disentangle the senescence of females from that of their male mates (and vice versa). For example, the reproductive success of females can be negatively affected by the poor ejaculate quality of old males (Dean *et al.*, 2010).

1.2.3. Intrinsic markers

In recent years there has been a growing integration of molecular, cellular and physiological markers (collectively termed intrinsic markers) of ageing into longitudinal field studies of ageing and life-history (e.g. Barrett *et al.*, 2013; Hayward *et al.*, 2015; Cheynel *et al.*, 2017). Intrinsic markers provide a means of characterising physiological senescence (i.e., within-individual changes in heath, functioning etc.) and, by extension, between-individual differences in patterns of senescence (i.e., biological age). They have also provided key insights into driving forces of differential senescence in wild populations (e.g., parasites, cooperative breeding, early-life environments etc.) that are not necessarily reflected in population-level demographic measures of senescence (i.e., survival and reproduction). Hence, intrinsic markers can reveal – what are often referred to as – "hidden" life-history costs (e.g. Asghar *et al.*, 2015). To date, the most widely utilized markers are oxidative stress and, more recently, telomere dynamics, due in part to their status as universal (inter-connected) mediators of senescence and life-history (López-Otín *et al.*, 2013). However, there are compelling arguments for the wider use of other promising intrinsic markers in field studies, such as IGF-1 levels (Lodjak and Verhulst, 2020), various blood parameters (Stier *et al.*, 2015) and epigenetic markers (Bell *et al.*, 2019; Simpson and Chandra, 2021).

There remain several uncertainties and challenges concerning the use and interpretation of intrinsic markers in ageing studies, even for those that are relatively well-validated in wild populations, i.e., oxidative stress and telomere dynamics (Speakman *et al.*, 2015; Young, 2018). Notably, the combined use of multiple intrinsic and demographic senescence markers has revealed high levels

of asynchrony among senescent traits within populations (e.g. Massot *et al.*, 2011; Hammers *et al.*, 2015; Hayward *et al.*, 2015; Gaillard and Lemaître, 2017), despite classic ageing theories predicting that the deterioration of biological functions with age should be coordinated (Williams, 1957; Smith, 1962). The reasons for this asynchrony are uncertain, but suggests that some traits, functions, or systems are better protected from senescence than others, perhaps because they are more important to maintain for Darwinian fitness (Nussey, Kruuk, *et al.*, 2009; Thomas *et al.*, 2016). Asynchrony could also arise from differing levels of adaptability in ageing mechanisms; while some may be relatively "fixed" (i.e. constraint-based) others are more easily "moulded" by selection and/or resource-allocation (Cohen *et al.*, 2020). Importantly, these case studies demonstrate the need to utilise several intrinsic markers, reflecting a variety of ageing hallmarks, to better understand the complexities of senescence in wild populations.

1.3. Intrinsic markers of senescence

In the following sections, I introduce several key intrinsic markers used in the study of senescence in wild populations. A full review of every potential marker (of which there are many) is beyond the scope of this introduction. Instead, I focus on markers that are of relevance to our study system (the Seychelles warbler) and this thesis, and which have wider relevance to other long-term longitudinal studies of wild taxa. For instance, I only include markers that reflect ageing mechanisms which, as far as we know, are conserved across taxa – so-called 'public' mechanisms (Partridge and Gems, 2002) – even though some markers relating to 'private' mechanisms may be of greater functional importance in their respective taxa; for example, rate of tooth wear in deer (Nussey *et al.*, 2007). Furthermore, I focus on markers that can be obtained through non-destructive, minimally-invasive means (namely blood sampling) as this is a constraint of longitudinal studies (Stier *et al.*, 2015). While references are made to findings from human and lab-based studies, there is an emphasis on existing evidence (or lack thereof) from wild and/or non-model taxa.

1.3.1. Oxidative stress

Reactive oxygen species (ROS) are generated by mitochondria during oxygen metabolism (Finkel and Holbrook, 2000). Normal levels of ROS, balanced by metabolic demands and antioxidant defences, act as intracellular signals involved in cellular homeostasis (Hekimi, Lapointe and Wen, 2011). However, oxidative stress occurs when high ROS levels are not neutralized by enzymatic antioxidants, causing damage to cellular structures and inducing apoptosis. A positive feedback loop can also occur when damage to mitochondrial DNA impairs the electron transport chain, reducing the respiratory efficiency of cells and causing further generation of ROS (Turrens, 2003). The free-radical theory proposes that an accumulation of this damage in cells/tissues contributes to biological ageing (Harman, 1965; but see Kirkwood and Kowald, 2012). Elevated ROS, and thus the potential for oxidative stress, occurs (broadly speaking) in response to increased physical

activity/metabolism (see Soulsbury and Halsey, 2018), stress exposure (Monaghan, 2014), growth, and reproduction (Blount *et al.*, 2016); thus, oxidative stress is a proposed mediator of early-late LHTs (Monaghan, Metcalfe and Torres, 2009; Speakman *et al.*, 2015).

Oxidative balance/status can be quantified as the ratio of ROS or Reactive Oxygen Metabolites (ROMs) versus antioxidant capacity (OXY) in Red Blood Cells (RBCs) or blood plasma. Since circulating blood is exposed to exogenous ROS generated from surrounding tissues, RBCs and plasma act as oxidative sinks, and thus are considered to reflect organismal oxidative state (Richards *et al.*, 1998; Kiefer and Snyder, 2000). However, metrics of oxidative stress solely from blood or other single tissues have received criticisms (Speakman *et al.*, 2015, see below).

There are several extensive reviews on the use of oxidative stress markers in the study of ageing and life-history in wild populations (Buffenstein *et al.*, 2008; Monaghan, Metcalfe and Torres, 2009; Speakman and Selman, 2011; Selman *et al.*, 2012; Speakman *et al.*, 2015). At the time, these reviews were motivated by the lack of consistent findings in the rapidly growing number of studies using oxidative markers and, more importantly, findings that questioned and/or directly challenged the importance of oxidative stress in ageing. These findings include (but are not limited to) a lack of a consistent relationship between oxidative status and age (Chen, Hales and Ozanne, 2007; Selman *et al.*, 2012; Stier *et al.*, 2015), no positive effects of antioxidants on longevity (Howes, 2006) and higher ROS production in longer-lived individuals/species (Andziak *et al.*, 2006; Pérez *et al.*, 2009). Associations with survival are also mixed; antioxidant capacity positively predicts survival in barn swallows (*Hirundo rustica*; Saino *et al.*, 2011) while the is no relationship between oxidative status and survival in Seychelles warblers (*Acrocephalus sechellensis*; van de Crommenacker *et al.*, 2011). Therefore, individuals may be able to avoid/mitigate the damage caused by oxidative stress (Edrey *et al.*, 2011; Sena and Chandel, 2012; Selman *et al.*, 2012). Alternatively, oxidative stress may only contribute to organismal ageing/survival by causing damage to certain key tissues/organs (e.g. the pancreas; Rashidi, Kirkwood and Shanley, 2009) which cannot be easily sampled through non-invasive/non-destructive means. Hence, the short-term oxidative status of more accessible single tissues (e.g. blood) may have little functional importance (Speakman *et al.*, 2015).

Nevertheless, an individual's current oxidative status could provide important insights into the somatic costs of life-history. For example, a meta-analysis found an overall positive association between reproductive effort (e.g. number of offspring being produced) and oxidative stress in females of various species (Blount *et al.*, 2016). However, this result was from observational, rather than experimental, studies. Metcalfe and Monaghan (2013) highlight that only by manipulating an individual's 'planned' reproductive effort can costs (in terms of elevated oxidative stress) be determined, since the naturally greater reproductive effort made by some individuals may reflect an enhanced ability of the individual to mitigate oxidative stress. In female black-legged kittiwake

(*Rissa tridactyla*), manipulating the sex-ratio of broods to male-biased (males being more energetically costly to feed) results in elevated oxidative stress (Merkling *et al.*, 2017). However, other brood manipulation studies have yielded conflicting results (e.g. Selman *et al.*, 2012 and references therein Merkling *et al.*, 2017). Fewer studies have examined the effect of growth rate on oxidative stress, though there is a positive relationship in both Soay sheep (Nussey *et al.*, 2009) and king penguins (*Aptenodytes patagonicus*; Geiger *et al.*, 2012). Therefore, oxidative status could reflect the costs of life-history investments on somatic maintenance.

1.3.2. Telomere dynamics

Terminal telomeres are repetitive nucleotide sequences located on the ends of chromosomes, which protect the functional integrity of the genome. Due to the 'end replication problem' (Watson, 1972), telomeres shorten with each cell division, until a critical length is reached where cells can no longer divide, which can trigger cellular senescence and/or apoptosis (Olovnikov, 1996; Campisi, 2003). Hence, telomere attrition can contribute to organismal senescence via the accumulation of senescent cells in tissues (Van Deursen, 2014). Oxidative stress is thought to be an important contributor to the rate of telomere attrition, with telomeres more exposed to the detrimental effects of ROS compared to the rest of the genome (Von Zglinicki, 2002; Reichert and Stier, 2017).

Telomeric DNA can be replaced by the enzyme telomerase and other mechanisms (Blackburn *et al.*, 1989; Davis and Kipling, 2005; Cesare and Reddel, 2010). Cell lines which require greater proliferation (e.g., male gametes) have greater telomerase activity (Gomes *et al.*, 2011), and artificially increasing telomerase activity leads to faster regeneration of some tissues (Reichert *et al.*, 2014). Telomerase downregulation in normal somatic tissue places a cap on cell proliferation, which may act to reduce the risk of tumour formation (Shay and Wright, 2011). Most telomere studies are based on blood samples, and both leukocytes and nucleated RBC telomere length are considered to be representative of whole organism telomere length (Daniali *et al.*, 2013; Reichert *et al.*, 2013). However, the relative abundance of circulating leukocyte cell-types can vary considerable within-individuals (e.g. due to infection) which could confound telomere length measurements (discussed in Nussey *et al.*, 2014).

Telomere length and dynamics have been used extensively in studies of wild populations as markers of both chronological- and biological-age (but see Dunshea *et al.*, 2011; Boonekamp *et al.*, 2013), LHTs (reviewed in Young, 2018) and individual quality (e.g. Angelier *et al.*, 2019). Numerous studies have demonstrated that telomere length declines with age within individuals (see Stier *et al.*, 2015). Furthermore, studies in humans, mice and wild bird species show that shorter telomeres and/or faster rates of telomere shortening are associated with higher mortality risk (Haussmann, Winkler and Vleck, 2005; Bize *et al.*, 2009; Heidinger *et al.*, 2012; Vera *et al.*, 2012; Barrett *et al.*, 2013).

Whether telomere dynamics are causally implicating in, or just correlated with, senescence (e.g. by reflecting long-term oxidative stress exposure) has been the subject of extensive discussion (Mikhelson and Gamaley, 2013; Simons, 2015; Young, 2018). Generally, there is greater support for the latter. For example, telomerase knockout and overexpression studies on laboratory organisms find weak evidence for telomere length determining lifespan (Simons, 2015). Furthermore, the power of telomere length to predict survival often diminishes in older ages (Heidinger *et al.*, 2012; Boonekamp *et al.*, 2013b), which is contrary to expectations if mortality was 'caused' by telomeres reaching critically short lengths (Simons, 2015). Therefore, telomere length more likely acts as a non-causal marker of general cellular damage caused by other mechanisms, such as oxidative stress (Reichert and Stier, 2017). For example, In king penguin chicks (*Aptenodytes patagonicus*), oxidative stress and telomere loss occur in tandem (Geiger *et al.*, 2012), and anti-oxidant defences are shown to prevent telomere attrition in dragon lizards (*Ctenophorus pictus*; Ballen *et al.*, 2012).

There are numerous factors in early-life and adulthood associated with faster telomere attrition. Within populations, accelerated telomere loss has been associated with higher reproductive effort (Bauch, Becker and Verhulst, 2012; Ouyang *et al.*, 2016), growth rate (Hall *et al.*, 2004; Foote *et al.*, 2011; Geiger *et al.*, 2012) and harsher environmental conditions (Watson, Bolton and Monaghan, 2015; Kirby, Alldredge and Pauli, 2017; Spurgin *et al.*, 2018). Furthermore, experimentally manipulating natal adversity results in accelerated telomere loss in juvenile birds and primates (Boonekamp *et al.*, 2014; Nettle *et al.*, 2015, 2017; Drury *et al.*, 2017).

Interestingly, telomere lengthening has also been observed in humans and several wild vertebrate species. (Svenson *et al.*, 2011; Fairlie *et al.*, 2016; Hoelzl *et al.*, 2016; Spurgin *et al.*, 2018; van Lieshout *et al.*, 2019). The mechanistic reasons behind telomere lengthening are not well understood but suggest that the costs of life-history (i.e., linked to telomere shortening) could be reversed in some circumstances. For example, in edible dormice (*Glis glis*) telomeres usually shorten post hibernation, but individuals provided with supplementary feed exhibit telomere lengthening (Hoelzl *et al.*, 2016). The phenomenon of telomere lengthening is investigated and discussed in detail in Chapter 3.

1.3.3. Cellular senescence, inflammation and immunosenescence

Normal somatic cells have a limited replicative lifespan, which is further shortened by stress-induced damage. By preventing the propagation of damaged cells, cellular senescence is a vital stage in the normal cellular turnover of tissues. However, tissue function declines as senescent cells accumulate with age (Wang *et al.*, 2009), due to an increased rate of senescent cell generation and/or a reduced capacity to remove and replace senescent cells with age, i.e. because of immunosenescence and replicative senescence (López-Otín *et al.*, 2013; Van Deursen, 2014). An accumulation of senescent cells, paralleled with immunosenescence, promotes chronic tissue

inflammation (known as 'inflammaging'; Deeks, 2011; Salminen, Kaarniranta and Kauppinen, 2012) that further aggravates cellular senescence and tissue damage (López-Otín *et al.*, 2013; Van Deursen, 2014).

Cellular senescence and associated mechanisms (i.e., inflammation and immunosenescence) are arguably better indicators of organismal senescence than oxidative stress or telomere length (which are themselves pathways to cellular senescence) since they are more causally implicated in the declining function of tissues and systems. In contrast, oxidative stress and telomere length may not (necessarily) cause wider damage, and there are a multitude of other stress-induced triggers of cellular senescence that operate independently (Van Deursen, 2014; Hernandez-Segura, Nehme and Demaria, 2018). However, measuring the abundance of senescent cells (which can be highly variable between tissue types (Wang *et al.*, 2009)) is challenging without the use of biopsies (reviewed in Hernandez-Segura, Nehme and Demaria, 2018). A more feasible marker (for use in longitudinal field studies) is proinflammatory cytokine levels (IL-6 and CRP; hereafter 'inflammatory markers') of blood plasma, which collectively reflects cellular senescence, inflammation and immune system dysregulation (López-Otín *et al.*, 2013; Fulop *et al.*, 2018).

In humans, inflammatory markers are positively associated with frailty in old age; based on a meta-analysis of 32 cross-sectional studies (Soysal *et al.*, 2016). Also in humans, increased adversity and intracellular damage in early-life are linked to increased adulthood inflammation (Danese *et al.*, 2007; Lacey, Kumari and McMunn, 2013; Nettle, 2014). Studies utilising inflammatory markers in wild taxa, though limited in number, concur with the findings from humans. Across populations of wild taxa, inflammatory markers increase with (cross-sectional) age (Peters *et al.*, 2019). For example, in both roe deer and Soay sheep, there is a paralleled increase in inflammatory markers and decrease in white-blood cell counts with age, indicating inflammaging and immunosenescence occur in tandem (Nussey *et al.*, 2012; Cheynel *et al.*, 2017). Furthermore, experimentally increasing natal-adversity causes increased adulthood inflammation in captive starlings (*Sturnus vulgaris*; Nettle *et al.*, 2017), which suggests inflammatory markers could also reflect early-late LHTs. However, despite their potential for the study of senescence in wild populations, inflammatory markers are yet to be widely used (Peters *et al.*, 2019).

1.3.4. Epigenetic clocks

DNA-Methylation - the most extensively studied epigenetic modification (Jones, Goodman and Kobor, 2015; Horvath and Raj, 2018) - is the binding of methyl groups (5-mC) to cytosine-guanine pairs (CpGs; Jones *et al.*, 2015), resulting in transcriptional silencing of genes. In humans, 70-80% of CpG sites are methylated (Bird, 2002). Methylation plays an essential role during organismal development through the regulation of gene expression (Bird, 2002). Both decreases and increases in methylation at certain CpGs can occur in a highly regular age-dependent manner (e.g. Horvath,

2013). Consequently, accurate estimates of chronological age (CA) can be calculated by incorporating several age-correlated CpGs (anywhere from 3 to *ca* 400 CpGs) into machine-learning predictive models (Horvath, 2013; Weidner *et al.*, 2014). So-called "DNA-methylation clocks" or "epigenetic clocks" have been developed in humans and a range of wild taxa; from European lobsters (*Homarus gammarus*; Fairfield *et al.*, 2021) to humpback whales (*Megaptera novaeangliae;* Polanowski *et al.*, 2014). In humans, clock-estimates of CA (hereafter epigenetic age) are considerably more accurate than those calculated from telomere length (Marioni *et al.*, 2016).

There are several lines of evidence to suggest that characteristics of DNA-methylation can reflect senescence in humans. Differences between epigenetic age and known CA inevitably occur due to individual-level variation in DNA-methylation. This difference – termed "epigenetic age acceleration" (EAA) – has been attributed to differences in biological age (BA). A higher epigenetic age, relative to CA (i.e. positive EAA), has been associated with poorer cognitive and physical performance, age-related diseases, higher mortality risk, and life-style factors associated with faster ageing, e.g. smoking (for review see Horvath and Raj, 2018). Furthermore, DNA-methylation clocks can be tailored for more direct and accurate estimates of BA by incorporating CpG-methylation that is associated with age-related health markers (e.g. white blood cell count) and/or mortality into predictive models (Zhang *et al.*, 2017; Levine *et al.*, 2018; Lu *et al.*, 2019). Whether methylation at clock-CpGs is also casually implicated in senescence (e.g. via dysregulated gene expression) remains uncertain (discussed in Horvath and Raj, 2018; Bell *et al.*, 2019).

There is some evidence to suggest DNA-methylation clocks also reflect BA in wild taxa (see Chapter 5 for more detail). For example, epigenetic ages are higher in males compared to females in roe deer and yellow baboons (*Papio cynocephalus*), which suggests DNA-methylation clocks could reflect the costs of male reproduction on ageing and lifespan in those species (Anderson *et al.*, 2021; Lemaître *et al.*, 2021; also see Sugrue *et al.*, 2021). However, no study of a wild population has yet assessed whether epigenetic age, or DNA-methylation generally, is directly associated with survival or lifespan. A possible limitation of DNA-methylation clocks for use in field studies is the financial cost of whole-genome methylation sequencing, which could make it unfeasible to apply DNA-methylation clocks to sample sizes large enough to investigate links with senescence and life-history. However, a promising cost-effective alternative is targeted sequencing of conserved candidate gene regions, for which age-associated DNA-methylation has been observed in other taxa. For example, Polanowski *et al.* (2014) developed the humpback whale DNA-methylation clock (for predicting CA) using orthologous CpGs/genes used in human and mice DNA-methylation clocks. However, suitable orthologous genes outside of mammals may be limited (e.g. De Paoli-Iseppi, Polanowski, *et al.*, 2017, for full discussion see Chapter 5).

1.3.5. Haematology

The intrinsic markers previously discussed can be measured from blood as this yields high qualities of DNA (from leucocytes or nucleated-erythrocytes) and tissue secretory products (in plasma) in one minimally-invasive sample (Stier *et al.*, 2015). Moreover, properties of blood components themselves can also provide useful markers of health and disease, i.e. the field of haematology in human medicine. Blood parameters (of which there are many) can be associated with human senescence. For example, fewer larger RBCs are observed in older individuals (Qiao *et al.*, 2014), and a higher RBC distribution width (i.e. variation in size) is associated with chronic inflammation, frailty and mortality (Martínez-Velilla *et al.*, 2015).

In field studies, blood parameters such as haematocrit and haemoglobin content – which are markers of blood oxygen-carrying capacity – are commonly used for assessing individual health, condition and disease, e.g. presence of blood parasites (Fair, Whitaker and Pearson, 2007; Minias, 2015; Johnstone, Lill and Reina, 2017). Since senescence is regarded as a decline in general health and/or increased incidence of disease with age – longitudinal changes in blood parameters could also be used to investigate senescence in wild taxa (reviewed in Stier *et al.*, 2015). However, despite the widespread use of blood parameters in field studies, few have investigated longitudinal change or associations with performance and/or survival in late-life (discussed in detail in Chapter 2).

1.3.6. Body mass

Body mass (relative to body size) is a routinely measured marker of body condition in most field studies. Variation in body mass, controlling for skeletal size, reflects an individual's energy reserves (fat/protein). The benefits of maintaining relatively high body mass are well documented. For example, a meta-analysis of several bird and mammal species showed that heavier offspring are more likely to survive to adulthood (Ronget, Gaillard, *et al.*, 2018). In old age, individuals may be less effective at acquiring/metabolising energy resources, resulting in a senescence of body mass. Within-individual declines in body mass with age and/or preceding death are particularly well documented in wild ungulate species (Gaillard *et al.*, 2000; Mysterud *et al.*, 2001; Nussey *et al.*, 2011; Hayward *et al.*, 2017). However, such declines may not be observed in other taxa; for example, the body mass of birds is highly constrained (optimised) as a result of flight requirements (e.g. Norberg, 1995). Nevertheless, body mass in early-life can reflect the quality of natal environment (Ronget, Gaillard, *et al.*, 2018), which is predicted to modulate senescence patterns (discussed in Chapter 4).

1.4. The Seychelles warbler study system

Figure 1. (top) An adult Seychelles warbler with colour rings for identification. (bottom left) Thomas Brown (me) taking routine measurements of a caught Seychelles warbler. (bottom right) The field research house on Cousin Island (Praslin Island in background).

1.4.1. Natural history

The Seychelles Warbler (SW) is a small insectivorous passerine bird endemic to the Seychelles archipelago. Historically, SWs occurred on several Seychelles Islands (Spurgin *et al.*, 2014). However, through anthropogenically induced habitat loss and the introduction of non-native predators, the SW population was reduced to less than 30 Individuals on Cousin Island (29 ha, 4°209 S, 55°409 E) in the 1960s (Loustau-Lalanne 1968). To save the species from extinction, the Island was made a nature reserve in 1968. Following the intensive restoration of native habitat, the population grew and stabilized. Since 1982, the population has been at carrying capacity; with *ca*

320 individuals in *ca* 115 territories (Komdeur, 1992; Komdeur and Pels, 2005; Hammers *et al.*, 2021). Translocations of individuals from Cousin have successfully established populations on four other Islands in the Seychelles, leading to the SW being downlisted from vulnerable to near-threatened in 2015 (Wright *et al.*, 2014). Regular monitoring of the Cousin SW population has been conducted since 1986 and has intensified since 1997 (Komdeur, 1992; Hammers *et al.*, 2015; Sparks *et al.*, 2020).

The SW is a facultative cooperative breeder. Socially monogamous breeding pairs (hereafter dominant pairs) defend territories year-round and normally stay together within the same territory for most of their lives (Komdeur, 1991; Richardson, Burke and Komdeur, 2007). Breeding can occur year-round but the majority of breeding activity coincides with seasonal peaks in food availability; the minor- and major breading seasons (January – March, June – September, respectively, Komdeur and Daan, 2005). Females usually produce a single-egg clutch, though two- or (very rarely) three-egg clutches from a single female do occur (Komdeur, 1991; Richardson *et al.*, 2001). There are high levels of extra-pair paternity, with *ca* 40% of offspring being sired by a dominant male from a different territory (Richardson *et al.*, 2001; Raj Pant *et al.*, 2019). Both the (social) dominant male and female contribute to the defence and provisioning of offspring, who remain dependent on parental care for *ca* four months (Komdeur, 1994). Offspring are sexually mature at 8 months and can obtain dominant breeding positions in their first year. However, due to population saturation (i.e. a lack of available dominant breeding positions), many independent offspring become subordinates of their natal territory; *ca* 50% of territories contain subordinates (Hammers *et al.*, 2019). *Ca* 30% of subordinates (both sexes) provide alloparental care to offspring of the dominant pair (Komdeur, 1994; Hammers *et al.*, 2019), and, in any given year, *ca* 40% of subordinate female helpers also gain reproductive success as co-breeders (Richardson *et al.*, 2001; Raj Pant *et al.*, 2019).

1.4.2. Data collection

Fieldwork is carried out for ca. six months of each year during the minor and major breeding seasons (Komdeur and Daan, 2005). During fieldwork, each territory is visited at least every two weeks to identify all individuals present and determine their status and breeding activity through behavioural observations (Richardson, Burke and Komdeur, 2003). Territory quality (the abundance of invertebrate prey on the underside of leaves) is also recorded for each territory per field season (see Komdeur, 1992 for details). Individuals are captured using mist nets and conspecific playback (see Kingma et al., 2016 for details). Since 1997, nearly all individuals (>96%) have been ringed with a unique combination of a British Trust for Ornithology (BTO) metal ring and three colour rings for identification (Richardson *et al.*, 2001; Raj Pant *et al.*, 2020). Individuals are usually first caught and ringed as nestlings or dependent fledglings (within ca 3 months of fledgling). In addition to capturing unringed juveniles, as much of the ringed adult population as possible (*ca* 40–60%) is re-captured

each year (Hammers *et al.*, 2015). The resighting probability of adults during the major breeding season is close to one (0.98 ± 0.01 SE; Brouwer *et al.*, 2010) and dispersal from the island is virtually absent (Komdeur *et al.*, 2004). Therefore, individuals that are not observed during the major breeding season can be confidently assumed dead.

During capture events, *ca* 70 µl of whole blood is drawn with a microcapillary tube from the brachial vein. A small amount (*ca* 10 ul) of blood sample is also stored in absolute ethanol at 4°C for future DNA extraction. This procedure is the routine, non-lethal way to sample blood from passerine birds and has been shown to have no measurable effect on condition or survival (Sheldon *et al.*, 2008). As well as taking blood samples, several biometrics are routinely recorded, including body mass (± 0.1g), tarsus length (± 0.1mm), head-bill length (± 0.1mm) and fat scores (Hammers *et al.*, 2015). The *ca* 70 µl of whole blood is centrifuged (to separate blood cells from plasma) and haematocrit is measured (± 0.01mm, see Chapter 2). In previous studies, blood plasma has also been used to measure testosterone levels and oxidative status (Van De Crommenacker *et al.*, 2004; Van de Crommenacker *et al.*, 2011). Extracted DNA is used to determine sex, parentage, the presence of malarial parasites and telomere length (see Chapter 3 for specific methods). The rare combination of a long-term individual-based dataset, with longitudinal data on multiple traits (including status, reproductive success, condition, malarial infection), known chronological age and a lack of migration (thus highly accurate survival estimates) provides the ideal study system for investigating senescence in a wild population. Furthermore, an extensive library of stored blood samples presents the opportunity to explore additional promising intrinsic markers of biological age.

1.4.3. Life-history and senescence

The patterns of demographic and physiological senescence in the Cousin SW population are well-documented (reviewed in Hammers *et al.*, 2015). The SW has an exceptionally long lifespan for a passerine, with the maximum recorded lifespan of 19 years old (Hammers and Brouwer, 2017) and average lifespan is 5.5 years; for individuals that reach fledgling age (Komdeur, 1991). Given that adult SWs lack predators, and the climate/weather is relatively benign, extrinsic mortality is much lower compared to other passerine systems. Annual survival is high and relatively stable at *ca* 85% from one to seven years-of-age. From the age of seven, annual survival progressive declines (Hammers *et al.*, 2015). There is little difference in survival senescence between males and females (Hammers *et al.*, 2015).

Interestingly, the onset and rate of survival senescence are associated with past and present reproductive investments. Individuals that become dominant breeders at a younger age (age-at-dominance ranging from 1 to 8 years-old) have an earlier onset of survival senescence, representing an early-late LHT (Hammers *et al.*, 2013). Furthermore, dominant females (but not males) with helpers in their territory have reduced survival senescence (i.e. higher annual survival from 7 years-

of-age) and reduced telomere attrition, relative to dominant females without helpers (Hammers *et al.*, 2019). Therefore, the presence of helpers, perhaps by contributing to offspring provisioning (van Boheemen *et al.*, 2019), appear to alleviate the costs of reproduction to somatic maintenance in old dominant females.

In both males and females, annual reproductive success increases within-individuals with age in early-life, likely reflecting gains in breeding experience (Hammers *et al.*, 2012; Raj Pant *et al.*, 2020). Peak annual reproductive success occurs at *ca* 6- and 8 years-of-age for females and males, respectively, above which annual reproductive success declines at similar rates for both sexes (Hammers *et al.*, 2012; Raj Pant *et al.*, 2020). In old dominant females (≥ 6 years-of-age), reproductive success is also lower in the last year of life (terminal effect; Hammers *et al.*, 2012). Similar to their effect on survival senescence, the presence of helpers reduces reproductive senescence (i.e., higher annual reproductive success from 6 years-of-age) in dominant females (Hammers *et al.*, 2021). This effect seems to be driven by helpers compensating age-related declines in dominant female provision rates (Hammers *et al.*, 2021).

Age-related changes in parasitic burden have also been investigated in the SW. The only known parasite to affect the SW is a strain of avian malaria (Hutchings, 2009). Prevalence of malaria is high among juveniles and young adults (*ca* 70%) but decreases up to the age of four (*ca* 20%; Hammers *et al.*, 2016a). This decrease occurs within-individuals (e.g. a clearing of chronic infection), rather than selective disappearance of individuals carrying the disease (Hammers *et al.*, 2016). While individuals can become re-infected (or have relapses in suppressed infection) in later-life, malaria prevalence does not increase with age in late-life, which suggests old individuals are not more vulnerable to infection or relapses (Hammers *et al.*, 2016). Although malaria prevalence is not associated with annual survival, dominant breeders have elevated oxidative stress when infected with malaria during chick provisioning (van de Crommenacker *et al.*, 2012). This suggests that malaria infection could aggravate the somatic costs of energy-demanding behaviours.

Oxidative status has been investigated in relation to several other factors. Individuals in poorer-quality territories (i.e. low food availability) have higher ROMs levels (Van de Crommenacker *et al.*, 2011). Furthermore, dominant males have elevated ROMs during mate-nest guarding (which is energetically costly) relative to other breeding stages (van de Crommenacker, Komdeur and Richardson, 2011). Taken together with the additive effect of malaria and chick provisioning, these results suggest oxidative status is indicative of where and when energy-expenditure is elevated in SW. Oxidative status may also be associated with the decision to engage in energy-demanding behaviours. For example, subordinates with higher ROMs do not participate in helping at the nest (van de Crommenacker, Komdeur and Richardson, 2011). However, in a pilot study there was no association between oxidative status (in the preceding year) and annual survival nor annual

reproductive success (van de Crommenacker *et al.*, 2011). It could be that oxidative status is simply too variable in the short-term to have long-term associations with fitness. Alternatively, SWs may be able to mitigate the potential damage from oxidative stress on somatic- and reproductive systems (see Hammers *et al.*, 2015 for discussion)

In the SW, telomere length declines within individuals with increasing age, and the rate of decline is greatest in early-life (Barrett *et al.*, 2013; Spurgin *et al.*, 2018). Furthermore, shorter telomere lengths and faster rates of shortening (controlling for age) are also associated with lower survival prospects (Barrett *et al.*, 2013). Therefore, the SW system conforms to classic telomere theories of ageing, i.e. that telomere shortening reflects the severity of senescence. Telomere length also reflects between-individual differences in body mass and body size. Heavier juveniles have longer telomeres, while heavier old individuals have shorter telomeres (Barrett *et al.*, 2013), and adult body size (tarsus length) is negatively associated with telomere length, independently of age (Spurgin *et al.*, 2018). Telomere length is also positively associated with temporal differences in island-wide food availability (Spurgin *et al.*, 2018). These somewhat contradictory results could indicate an early-late LHT between the benefits of high food availability and being heavier in early-life – resulting in long telomeres – and delayed costs of growth (i.e. being heavier and larger) to somatic maintenance in late-life – resulting in short telomeres (Hammers *et al.*, 2015).

In addition to age-dependent shortening, within-individual telomere dynamics also appear to reflect differing life-history costs and/or stress. Dominant males living in territories with a greater number of rival neighbouring males (i.e. increased social conflict) have faster rates of telomere shortening (Bebbington *et al.*, 2017). Dominant females with helpers in their territory have reduced rates of telomere shortening, relative to dominant females without helpers (Hammers *et al.*, 2019). However, telomere dynamics are highly variable within individual SWs, which as yet remains largely unexplained (Hammers *et al.*, 2015). Of particular interest, telomeres frequently increase in length within individuals; a phenomenon that has only recently been observed and recognised in wild populations (Spurgin *et al.*, 2018). Some telomere lengthening (and variation in telomere length generally) originates from technical error in PCR-based measurements (Sparks *et al.*, 2021). However, the frequency and strength of telomere lengthening are greater than expected from measurement error alone (Spurgin *et al.*, 2018). The circumstances under which telomere lengthening occurs in wild populations are largely unknown (see Chapter 3).

1.5. References

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Chapter 2
Haematocrit, age and survival in a wild vertebrate population

Seychelles warblers painted by Sabrina Das (top left) and Thomas Brown (bottom right)

2.1. Abstract

Understanding trade-offs in wild populations is difficult, but important if we are to understand the evolution of life histories and the impact of ecological variables upon them. Markers that reflect physiological state and predict future survival would be of considerable benefit to unravelling such trade-offs and could provide insight into individual variation in senescence. However, currently used markers often yield inconsistent results. One underutilised measure is haematocrit, the proportional of blood comprising of erythrocytes, which relates to the blood's oxygen-carrying capacity and viscosity, and to individual endurance. Haematocrit has been shown to decline with age in cross-sectional studies (which may be confounded by selective appearance/disappearance). However, few studies have tested whether haematocrit declines within-individuals or whether low haematocrit impacts survival in wild taxa. Using longitudinal data from the Seychelles warbler (*Acrocephalus sechellensis*), we demonstrated that haematocrit increases with age in young individuals (<1.5 years) but decreases with age in older individuals (1.5–13 years). In breeders, haematocrit was higher in males than females and varied relative to breeding stage. High haematocrit was associated with lower survival in young individuals, but not older individuals. Thus, while we did not find support for haematocrit as a marker of senescence, high haematocrit is indicative of poor condition in younger individuals. Possible explanations are that these individuals were experiencing dehydration and/or high endurance demands prior to capture, which warrants further investigation. Our study demonstrates that haematocrit can be an informative metric for life-history studies investigating trade-offs between survival, longevity and reproduction.

2.2. Introduction

An organism's fitness is the product of many integrated physiological systems, and their interaction with the environment. Activity in one physiological system can limit resource availability and generate negative consequences (e.g. by-products) for another (Leroi, 2001; Ricklefs and Wikelski, 2002; Harshman and Zera, 2007). These trade-offs form the basis of life-history. Physiological markers provide valuable insights into life-history trade-offs, condition and senescence - particularly in wild populations, where complex environmental factors can weaken associations between life-history traits and observed fitness (Nussey *et al.*, 2008). Many physiological markers (oxidative stress, hormone regulation etc.) yield complex and/or inconsistent associations with life-history, survival and reproductive success (e.g. Norris and Evans, 2000; Speakman and Selman, 2011; Wilder, Raubenheimer and Simpson, 2016; Johnstone, Lill and Reina, 2017), thus there is a continued need to identify and validate such markers.

Aerobic capacity, which contributes to endurance and performance, is a vital physiological trait for organismal health and fitness. Aerobic capacity depends on the oxygen-carrying capacity of blood, which is determined by the concentration of haemoglobin and the rate of blood flow, which is

inversely proportional to blood viscosity (Wagner, 1996; Birchard, 1997; Calbet *et al.*, 2006). These properties are reflected by haematocrit or Packed Cell Volume (PCV); the proportion of whole blood volume comprised of erythrocytes. Haemoglobin and blood viscosity increases linearly and exponentially, respectively, with haematocrit (Hedrick et. al., 1986). Blood becomes harder to circulate with increasing viscosity (i.e. requiring greater cardiovascular effort), but less viscous blood contains less haemoglobin. Therefore, intermediate haematocrit levels (ca. 40%) are optimal for maximum oxygen carrying capacity and endurance (Birchard, 1997; Schuler *et al.*, 2010; Jensen *et al.*, 2013).

Haematocrit levels observed in nature are variable (range ca. 30–60%) within and between endothermic species (Stark and Schuster, 2012). For example, haematocrit is higher in species/individuals requiring greater blood oxygen storage and endurance (Lourdais et al., 2014; Minias, 2015; Yap, et al., 2019). Similarly, haematocrit tends to increase within-individuals in response to elevated oxygen demands, such as during altitudinal migration (Borras et al., 2010) and exercise regimes (reviewed in Yap, et al., 2017). Elevated haematocrit occurs via the production of new erythrocytes (erythropoiesis) and/or the release of reticulocytes (immature erythrocytes) from the bone marrow, which is triggered by hypothalamus-pituitary-adrenal mediated stress (see Voorhees et al., 2013). More rapid (< 1 hour) increases in haematocrit can occur due to a reduction in blood plasma volume (haemoconcentration), which happens during exercise and dehydration (Kaltreider and Meneely, 1940; Bury *et al.*, 2019). In some mammal species, splenic reservoirs of erythrocytes can also increase haematocrit at the onset of stress and exercise (Böning, et al., 2011).

Anaemia – characterised by chronically low haematocrit and haemoglobin – occurs when an individual's rate of erythrocyte loss exceeds that of erythropoiesis, for example, during blood parasitism (O'Brien et al., 2001). However, anaemia can occur without affecting haematocrit, since the release of reticulocytes, which are larger than mature erythrocytes, can rapidly complement haematocrit despite them having lower haemoglobin content (Fair et al., 2007). Anaemia can also arise as a secondary outcome of competing physiological systems. For example, egg-production in birds causes a reduction in haematocrit via an oestrogen-mediated suppression of erythropoiesis and haemodilution – an increase in blood plasma volume (Williams et al., 2004; Wagner, et al., 2008). Therefore, both within-individual increases and decreases in oxygen carrying capacity and associated factors (haematocrit and haemoglobin) have the potential to reflect a multitude of life-history events and trade-offs (for reviews see Fair, et al., 2007; Minias, 2015; Johnstone, et al., 2017).

Uncertainty remains regarding associations between haematocrit, age and senescence in wild animals. From birth to maturity, haematocrit increases with age (e.g. Eklom and Lill, 2006; Trillmich, et al., 2008; Cornell and Williams, 2017), but few studies have determined the age-dependence of

haematocrit in adult-life. This likely stems from the difficulty of obtaining samples of known-age adults in many wild systems. In captive mice and humans, low haematocrit in extreme old age reflects senescence in erythrocyte renewal mechanisms (Boggs and Patrene, 1985; Gaskell *et al.*, 2008). Similarly, cross-sectional studies of other captive and wild vertebrates have observed lower haematocrit in old-age, suggestive of senescence (Smucny et al., 2004; Prinzinger and Misovic, 2010; Jégo et al., 2014; Elliott et al., 2015). However, such observations may also arise from compositional changes in successive age classes of a population e.g. due to selective disappearance of individuals with high haematocrit. Longitudinal studies are needed to explicitly investigate within-individual change with age (Nussey et al., 2008; Elliott et al., 2015).

Factors that cause haematocrit to deviate from the theoretical optimum (for oxygen-carrying capacity and general health) could have long-term impacts on the fitness of wild taxa. For example, experimental reductions of haematocrit in birds can result in reduced reproductive success (Fronstin et al., 2016) and flight performance (Yap *et al.*, 2018). However, few studies have investigated associations between haematocrit levels observed under natural conditions in the wild and subsequent survival. Anaemia results in lethargy and fatigue, but even minor decreases in oxygen carrying capacity could represent an energetic disadvantage that reduces survival prospects in wild settings. Conversely, more viscous blood, and the cardio-vascular loading this creates, is linked to negative health impacts in humans (Stack and Berger, 2009; Brækkan *et al.*, 2010; Coglianese *et al.*, 2012; Walton *et al.*, 2017). Extreme high or low haematocrit can also be a non-causal indicator of factors detrimental to self-maintenance, such as stress, parasitism and nutrient deficiencies (see Johnstone et al., 2017). Therefore, intermediate haematocrit levels are expected to be optimal for survival (e.g. Boffetta et al., 2013; Bowers et al., 2014).

The isolated Seychelles warbler (*Acrocephalus sechellensis*) population on Cousin Island provides an excellent model system for studying associations between haematocrit, age and survival in a wild population. This system benefits from over 30 years of continuous monitoring and extremely accurate survival estimates of known-age individuals that are not confounded by dispersal (Komdeur, 1992; Richardson et al., 2007; Hammers et al., 2019). Individuals have been captured and blood sampled repeatedly across their life-time, providing a wealth of longitudinal physiological data (Hammers *et al.*, 2015), including haematocrit. Here, we first assess the relationship between haematocrit and age. Based on previous findings across vertebrate taxa, we predict that haematocrit increases during early-life up to maturity, followed by an age-related decline. Crucially, we determine the relative contribution of longitudinal (i.e. within-individual) and cross-sectional (i.e. between-individual) effects to any age-patterns observed. Haematocrit is also likely to vary between and within individuals independently of age. We determine whether this variation is explained by other factors, namely sex and social status and breeding stage, and assess within-individual repeatability of haematocrit. Lastly, we determine the relationship between haematocrit

and annual survival probability. Given the potentially negative effects of both low and high haematocrit, we predicted that individuals with intermediate haematocrit values would have higher survival. Our study will, therefore, assess the validity of haematocrit as a marker of condition within wild animal populations, and explore its usefulness in terms of providing insights into the costs and trade-offs that individual animals face during life.

2.3. Methods

2.3.1. Study species and data collection

The Seychelles warbler is a small insectivorous passerine endemic to the Seychelles. Seychelles warblers can (exceptionally) reach ages of up to 19 years old (Hammers and Brouwer, 2017), though the average lifespan is 5.5 years for individuals that reach fledgling age (Komdeur, 1991). The population of ca. 320 adult individuals on Cousin Island (29 ha, 4°209 S, 55°409 E) has been extensively monitored since 1986. Monitoring is carried out for ca. 6 months of each year (January–March, June – September) during the minor and major breeding seasons, respectively (Komdeur and Daan, 2005). Since 1997, nearly all individuals (> 96%) have been ringed with a unique combination of a British Trust for Ornithology (BTO) metal ring and three colour rings for identification (Richardson *et al.*, 2001). Individuals are usually first caught and ringed as nestlings or dependent fledglings; before sexual maturity (< 8 months old). Juveniles are assigned to age categories (fledgling 1–3 months, old fledgling 3–5 months or sub-adult 5–8 months), based on behaviour and eye-colour, which transitions from grey in fledglings to red-brown in adults (Komdeur, 1992).

The population is structured into clearly defined territories that are defended year-round. Breeding groups comprise of one socially monogamous dominant pair (hereafter dominant breeders), but may also include 1–5 sexually mature subordinates (Richardson et al., 2002) which sometimes engage in helping behaviour and co-breeding (Hammers et al., 2019). An Individual's social status in a given field season is determined through observations of behaviour (see Komdeur, 2001; van de Crommenacker et al., 2011).

During the breeding season each territory is visited at least every two weeks and checked for the presence-absence of individuals identified by their colour ring combination. Dominant females are followed for 15 minutes to determine whether an active nest is present. Once a nest is found it is visited every 3 days for 15–60 minutes (to determine breeding stage) until completion or failure. For nests that were discovered during or after the start of incubation, the egg-laying date is estimated from the timing of hatching (determined from provisioning observations) and/or fledging. Given that inter-island dispersal is exceptionally rare (Komdeur et al., 2004) and resighting probabilities are close to one (Brouwer et al., 2009), birds that are not seen during a field season

can be assumed dead (Hammers *et al.*, 2013). The last day of a field season for which an individual is observed as present is taken as the date of death.

Individuals were captured using mist nets and conspecific playback (see Kingma et al., 2016 for details). Ca. 70 µl of blood was drawn with a microcapillary tube from the brachial vein. A small amount (ca. 10 ul) of blood sample was also stored in absolute ethanol at 4°C for future DNA extraction. This procedure is the routine, non-lethal way to sample blood from passerine birds and has been shown to have no measurable effect on condition or survival (Sheldon *et al.*, 2008). Within ca. three hours of bleeding, microcapillary tubes were centrifuged for 8 minutes at 8000 rpm to separate erythrocytes from plasma, white blood cells and platelets. Haematocrit was measured (using sliding callipers ± 0.01 mm) as the proportion of erythrocytes relative to whole-blood volume. Between the years of 2003 – 2017, 1383 haematocrit measurements were obtained from 733 individuals. DNA was extracted using a salt extraction technique following Richardson et al. (2001b) and sex of the individual was confirmed using the PCR-based method outlined by Griffiths et al. (1998).

2.3.2. Statistical analyses

All statistical analyses were performed with RStudio (v1.2.5033, RStudio team, 2020). Firstly, we investigated the relationship between haematocrit and age across all samples with a Generalized Additive Mixed Model (GAMM) using the gamm4 package (v0.2–6). In this model, we fitted a non-parametric smoothing parameter for age to evaluate expected non-linear relationships between haematocrit and age. Compared to Linear Mixed Models (LMMs), which require pre-specified functions between dependent and continuous predictor variables, GAMMs are more appropriate when the shape of age-dependent patterns are unknown (Hammers *et al.*, 2016). In addition to age, the model included factors known to influence haematocrit in avian taxa (see Fair et al., 2007); sex, social status (dominant breeders versus subordinates + juveniles) and time of day of sampling. Sex differences are likely to depend on social status; thus, a two-way interaction between sex and status was included. To control for non-independent samples, individual identity, breed group identity and catch year were included as random intercepts.

Age-related patterns across populations can arise from selective disappearance, whereby certain phenotypes are associated with shorter life-spans (Nussey *et al.*, 2008). To control for selective disappearance effects, we repeated the model using only individuals that were dead at the time of analysis and included age-at death as an additional factor (van de Pol and Verhulst, 2006; Hammers *et al.*, 2019).

Our GAMM analysis revealed that dominant females had significantly lower haematocrit than dominant males and subordinates (male or female). This suggested an effect of reproductive anaemia on haematocrit levels of dominant females, since they produce the majority of offspring

and sampling coincided with the breeding seasons. To determine whether sex-by-status differences were maintained in individuals not engaged in reproduction, we repeated the model including only sexually mature individuals (>8 months old) sampled outside of known breeding attempts; either no egg was laid for that breed group or the individual was sampled >50 days from the breed groups lay date. For a given individuals breed group, we calculated the number of days between the estimated lay date and the date of sampling. For breed groups with two or more broods (which occurs if, for example, the first brood was predated) the closest lay date from the sample date was selected. For individuals sampled during breeding attempts (<50 days from breed groups lay date), we expected haematocrit to be lowest nearer the lay date, and only in dominant females. Since haematocrit was expected to fluctuate non-linearly across breeding stages, non-parametric smoothing parameters were fitted for days from lay date for males and females. Separate models were created for dominant breeders and subordinates to avoid the need for complex three-way interactions between sex, status and days from lay date. Individuals were rarely caught multiple times within the same breeding attempt; thus this section of our analysis is cross-sectional in nature.

To separate the role of between- versus within-individual variation with age (i.e. cross-sectional from longitudinal effects), we used the within-subject centering method described by van de Pol and Wright, 2009. Briefly, age at sampling is split into two predictors, (i) mean age across all sampling events for a given individual (mean age), and (ii) within-individual deviation from mean age (Δ age). Our GAMM model indicated a peak in haematocrit at ca. 1.5 years of age (see results, Fig. 1). To investigate the initial increase and subsequent decrease in haematocrit in more detail, we performed within-individual centering for individuals <1.5 months and for individuals ≥1.5 months in separate analyses (following Hammers et al., 2016). This allowed us to compare the drivers of age-related haematocrit patterns in early life versus later adulthood.

We created Linear Mixed Models using the lme4 package (v1.1-21, Bates et al., 2014) with haematocrit as the response and mean age, Δ age, sex, social status and time of day of sampling as predictors. Age terms were entered as both linear and quadratic terms to test for possible non-linear patterns. Two-way interactions between Δ age, sex and status were included to determine whether within-individual changes in haematocrit were dependent on these factors. Consistent with the GAMMs outlined above, individual identity, breed group identity, and catch year were included as random intercepts. Due to the relationship observed between haematocrit and breeding stage in dominant breeders (see Fig. 2), we repeated the analysis excluding samples from breeding stages where haematocrit deviates from typical levels; 20 days before to 5 days after laying for dominant males, and 30 – 50 days after laying in dominant females. Using the rptR package (v0.9.22; Nakagawa and Schielzeth, 2010) we also calculated repeatability estimates for

haematocrit within-individuals to determine how consistent individual haematocrit levels are across repeated samples at different times.

Lastly, we investigated whether haematocrit predicts short-term survival. We used a binominal Generalised Linear Mixed Models (GLMM) to test the probability of surviving one year beyond the date of sampling (Y/N) in relation to haematocrit. Since haematocrit exhibited different age-specific patterns in early-life (<1.5 years) and adulthood (1.5–13 years; see results), we investigated the relationship between haematocrit and survival for these two age groups in separate models. Where multiple haematocrit samples were taken per individual, only the last sample was selected, which allowed us to identify whether individuals facing imminent mortality have different haematocrit levels compared to those which survive. Additional fixed effects included age, sex, status, and quadratic functions of haematocrit and age. We also included an interaction term between age and haematocrit to see whether the effect of haematocrit on survival changed with age. Survival probability can vary between territories and years e.g. due to varying food availability (Brouwer *et al.*, 2006; Spurgin *et al.*, 2017); thus, breed group and catch year were also entered as random factors. Breed group was subsequently dropped as a random factor in the 1.5–13-year group due to model convergence issues. As with the LMM analysis, we repeated models excluding samples from breeding stages where haematocrit deviates from typical levels in dominant breeders.

In all models, non-significant interaction terms were removed sequentially (in order of least significance) and only reported if of specific interest. All fixed effects remained in final models (regardless of significance) except for quadratic functions of continuous variables, which were removed when non-significant (see Whittingham et al., 2006). Parameter estimates and significance of removed effects were determined by re-entering them into final models.

2.4. Results

2.4.1. Cross-sectional age

Haematocrit had a distinctive pattern with cross-sectional age (Fig. 2.1). Initially, haematocrit increased rapidly before reaching a peak at ca. 1.5 years of age. From 1.5 years onwards (maximum age in this analysis is 13 years), haematocrit showed a consistent downward trajectory (Fig. 2.1). This age-dependent pattern was similar for both sexes (Fig. 2.1) and fitting smoothed age terms for males and females separately resulted in poorer model fit ($\Delta AIC > 4$). Sex-differences in haematocrit were dependent on social status (Table 2.1). For dominant breeders, which are the vast majority of individuals sampled ≥2 years-of-age, females had lower average haematocrit levels than males. Dominant females also had lower haematocrit than subordinates (male or female; Table 2.1, Fig S2.1). Individuals sampled in the early morning had higher haematocrit than those sampled in the late afternoon (Table 2.1). When the model was run on a sub-set of individuals known to be dead,

including age-at-death as a predictor, we found that shorter-lived individuals had significantly higher haematocrit (Table S2.1). Therefore, selective disappearance of individuals with high haematocrit contributed to the age specific pattern. Crucially, the effect of age was still significant when controlling for age-at-death (Table S2.1), indicating that within-individual effects were also present.

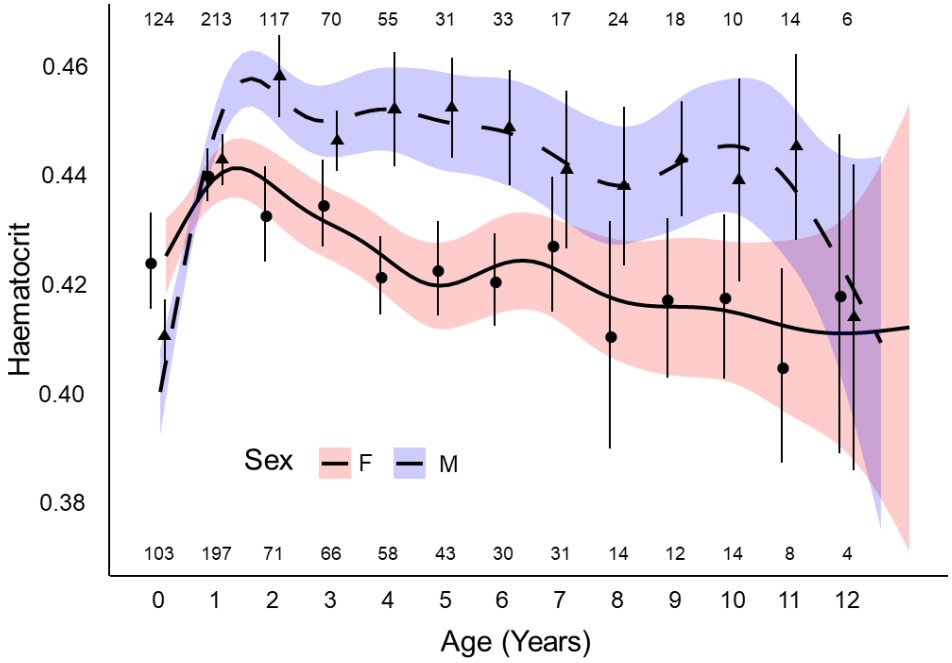

Figure 2.1: Haematocrit in Seychelles warblers in relation to age and sex. The fit lines (solid = female, dashed= male) show nonparametric smoothing functions for age with 95% confidence intervals. Points (round = female, triangles= male) are means and 95% confidence intervals for each age (rounded years). Ages 12 and 13 are grouped for graphical purposes (denoted as 12 here). Within-graph numbers represent sample sizes per age for females (lower) and males (upper).

Table 2.1: Haematocrit in relation to cross-sectional age and other factors in Seychelles warblers. Results are from a GAMM analysis with a non-parametric smoothing parameter for age. Significant effects are in bold.

Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.460	0.005	94.701	< 0.001
Sex (Male)	-0.003	0.003	-1.202	0.230
Status (Dominant)	**-0.021**	**0.003**	**-6.440**	**< 0.001**
Sample Time	**-0.002**	**< 0.001**	**-5.981**	**< 0.001**
Sex × Status	**0.028**	**0.004**	**7.612**	**< 0.001**
Smoothed Terms		***df***	**F**	***P***
Age		**7.805**	**16.73**	**< 0.001**
Random factors	**1379 observations**	**Variance**		
Individual identity	730 individuals	< 0.001		
Breed group	747 breed groups	< 0.001		
Catch year	14 years	< 0.001		

2.4.2. Reproductive stage

We compared haematocrit of sexually mature (>8 months-old) subordinates and dominant breeders. Outside of breeding attempts, dominant females had lower haematocrit than both dominant males and subordinate males and females (Table S2.2, Fig, S2.1). During breeding attempts, the effects were more complex (Table S2.3). There was no evidence of reproductive anaemia (i.e. a marked decrease in haematocrit) in dominant females sampled near their lay date, although haematocrit was lower at ca. 35–50 days after laying (Fig. 2.2). The haematocrit of males exhibited a complex relationship with breed group lay date; haematocrit was highest at ca. 7 days prior to laying and was lowest 15 – 30 days post laying (Fig. 2.2). In contrast to dominant breeders, there was no significant difference between male and female subordinates sampled during breeding attempts (Table S2.3 and S2.4, Fig. 2.2). The haematocrit of subordinate males did not vary in relation to breed group lay date, but subordinate females exhibited a weak quadratic relationship with days from breed group lay date; peaking at the laying date (Fig. 2.2). Importantly, the decline of haematocrit with increasing age persisted when the analysis was split between non-breeding and breeding individuals, and (for the latter) when controlling for days from lay date (Fig. S2.2).

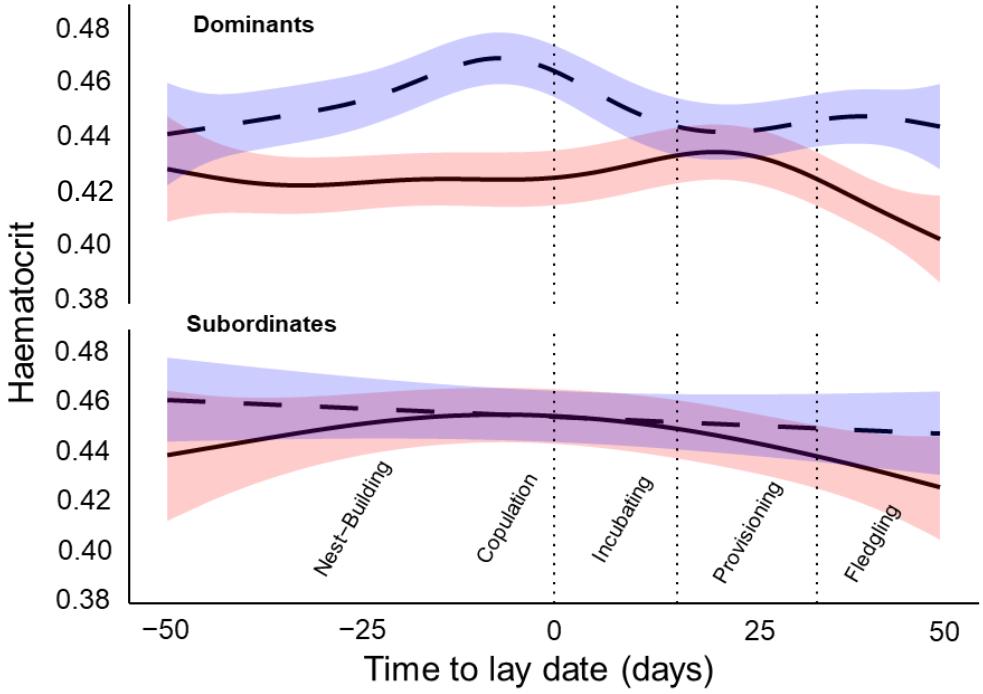

Figure 2.2: Haematocrit in in Seychelles warblers in relation to days from lay date for dominant breeders (left) and subordinates (right). The fit lines (solid = female, dashed = male) show non-parametric smoothing functions for days from lay date and the shaded area is the 95% confidence interval for the smoothing functions. The annotations and dotted lines denote theoretical nest stages relative to lay date.

2.4.3. Longitudinal age

Our within-subject centering analysis, which separates within- and between-individual contributions to age-patterns, was consistent with the GAMM analysis. Below 1.5 years of age, haematocrit increased both within- and between-individuals with age in a quadratic pattern; a strong initial increase which plateaued at ca. 1 year-of-age (Table 2.2, Fig. 2.3). From 1.5 years of age onwards, haematocrit declined linearly with increasing age both within- and between-individuals (Table 2.2, Fig. 2.3). All interactions with Δ age were non-significant; thus, within-individual increases (<1.5 years) and decreases (1.5—13 years) did not vary between individuals of differing sex or status. Consistent with the GAMM analysis, haematocrit was lower in dominant females and individuals caught later in the day (Table 2.2). All results were qualitatively identical when samples from dominant breeders caught during key breeding stages (where haematocrit deviated from typical levels; see fig 2.2) were excluded from the analysis (Table S2.5).

For final LMMs (excluding all non-significant interactions and non-significant quadratic effects), we calculated repeatability of haematocrit within-individuals. For individuals below 1.5 years-of-age, only 1.6% (P = 0.434) of variance was due to within-individual consistency. From 1.5 years-of-age, within-individual consistency was higher at 7.8% and approaching significance (P = 0.063).

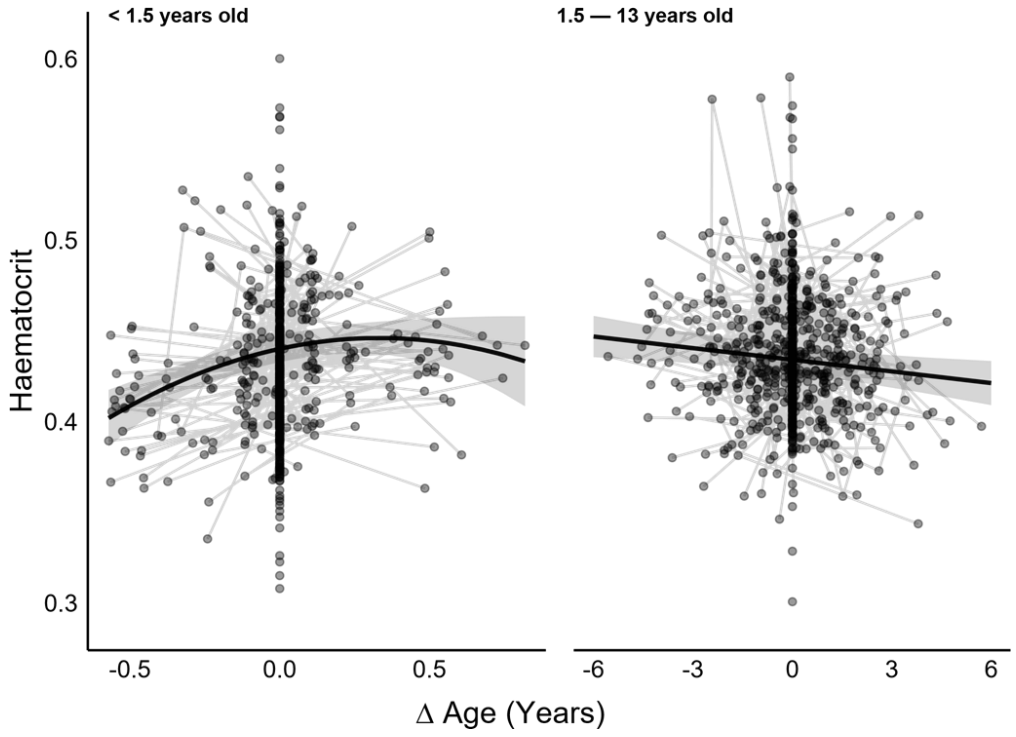

Figure 2.3: Haematocrit in Seychelles warblers in relation to within-individual differences in age (Δ Age) for individuals <1.5 years old and 1.5–13 years old. Thick black lines are the LMM (Table 2) predicted haematocrit ± 95% CI relative to age. Raw data are haematocrit samples with thin grey lines connecting multiple samples from the same individual.

Table 2.2: Haematocrit in relation to cross-sectional age (mean age) and longitudinal age (Δ age) in Seychelles warbler <1.5 years old and 1.5–13 years old. Parameters shown are from LMM analysis. Significant effects are in bold.

< 1.5 YEARS OLD				
Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.393	0.010	40.92	< 0.001
Mean Age	**0.159**	**0.022**	**7.327**	**< 0.001**
Mean Age^2	**-0.085**	**0.016**	**-5.436**	**< 0.001**
Δ Age	**0.036**	**0.008**	**4.437**	**< 0.001**
Δ Age^2	**-0.054**	**0.018**	**-3.09**	**0.002**
Sex (Male)	-0.005	0.003	-1.615	0.107
Status (Dominant)	**-0.014**	**0.007**	**-2.061**	**0.040**
Sample Time	**-0.002**	**<0.001**	**-3.276**	**0.001**
Sex × Status	**0.017**	**0.008**	**2.143**	**0.033**
Random factors	**637 observations**	**Variance**		
Individual identity	506 individuals	< 0.001		
Breed group	452 breed groups	< 0.001		
Catch year	14 years	< 0.001		
1.5 - 13 YEARS OLD				
Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.465	0.006	74.692	< 0.001
Mean Age	**-0.002**	**0.001**	**-3.608**	**< 0.001**
Δ Age	**-0.002**	**0.001**	**-2.715**	**0.007**
Sex (Male)	**0.026**	**0.002**	**10.677**	**< 0.001**
Status (Dominant)	**-0.013**	**0.003**	**-3.619**	**< 0.001**
Sample Time	**-0.002**	**0.001**	**-4.714**	**< 0.001**
Random factors	**742 observations**	**Variance**		
Individual identity	405 individuals	< 0.001		
Breed group	529 breed groups	< 0.001		
Catch year	14 years	< 0.001		

2.4.4. Survival

A total of 263 out of the 1383 samples taken were from individuals that died within the subsequent year. For young (<1.5 years-of-age), individuals with higher haematocrit were less likely to survive to the next year (Table 2.3, Fig. 2.4). In contrast, haematocrit did not predict survival over the subsequent year for individuals 1.5–13 years-of-age (Table 2.3). Contrary to expectations, there was no quadratic effect of haematocrit on survival; only high haematocrit was associated with lower survival in young individuals. The effect of haematocrit on survival was not influenced by age in either age category. Survival probability was lower for males and subordinates from 1.5–13 years-of-age. Repeating the analysis while excluding samples from dominant breeders caught at key breeding stages (where haematocrit deviated from typical levels; Fig 2.2) did not qualitatively change results (Table S2.6).

Table 2.3: Survival in the Seychelles warbler in relation to haematocrit for individuals 1.5 years old and 1.5–13 years old. Results are from binominal GLMMs with survival to the following year (Y/N) as the response variable. Significant effects are in bold.

< 1.5 YEARS OLD				
Predictor	**β**	**SE**	***z***	***P***
(Intercept)	5.016	1.283	3.909	< 0.001
Haematocrit	**-9.293**	**3.004**	**-3.093**	**0.002**
Sex (Male)	-0.224	0.224	-1.001	0.317
Status (Dominant)	0.565	0.369	1.533	0.125
Age	0.459	0.395	1.161	0.246
Random factors	**506 observations**	**Variance**		
Breed group	418 breed groups	< 0.001		
Catch year	14 years	0.340		
1.5 - 13 YEARS OLD				
Predictor	**β**	**SE**	***z***	***P***
(Intercept)	1.485	1.668	0.89	0.3733
Haematocrit	-1.398	3.684	-0.379	0.7044
Sex (Male)	**-0.557**	**0.254**	**-2.196**	**0.0281**
Status (Dominant)	**0.724**	**0.345**	**2.099**	**0.0358**
Age	-0.009	0.043	-0.202	0.8403
Random factors	**408 observations**	**Variance**		
Catch year	13 years	0.370		

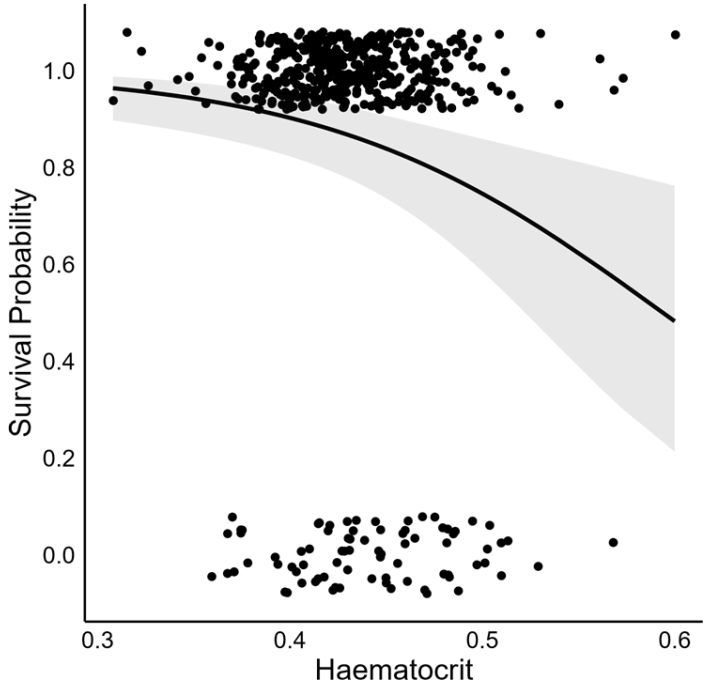

Figure 2.4: The probability of young (< 1.5 years of age) Seychelles warblers surviving one year after sampling relative to haematocrit. The fit-line is a binomial regression between survival (Y/N) and haematocrit with 95% confidence limits. Raw data depicts the distribution of observed survival counts (1 = survived, 0 = deceased).

2.5. Discussion

Haematocrit increased in juveniles up to 1.5 years-of-age, beyond which haematocrit declined with increasing age. Both longitudinal change and the selective disappearance of individuals with high haematocrit contributed to this age-specific pattern. In early-life (<1.5 years), haematocrit increased within-individuals, but individuals with higher haematocrit were less likely to survive to the following year. After 1.5 years of age haematocrit declined within-individuals with advancing age. However, haematocrit did not predict survival in this older age range. In addition to age, haematocrit was lower in females compared to males, but only in dominant breeders (haematocrit did not significantly differ between subordinates and dominant males). The haematocrit of dominant breeders also varied with breeding stage – most notably, male haematocrit peaked in the days prior to the pair-bonded females lay date. However, the relationship between haematocrit with both age and survival persisted when samples from dominant individuals caught during key breeding stages were excluded from analyses.

2.5.1. Age

Increases in haematocrit and oxygen carrying capacity during development have been observed in a range of vertebrates (Petschow et al., 1978; Fair et al., 2007; Trillmich et al., 2008). In birds, adult levels of haematocrit are usually achieved at late-nestling age, presumably in preparation for fledging (Eklom and Lill, 2006a, 2006b). However, in the Seychelles warbler haematocrit continues to increase post-fledging (up to 1.5 years). Our longitudinal and survival analyses confirmed that increases in haematocrit occurred within juveniles and were not due to juveniles with low haematocrit having lower annual survival i.e. selective disappearance. In this species, fledglings receive parental care (provisioning) for up to 3 months (Komdeur, 1996) and may delay dispersal from the natal territory (i.e. become subordinates) for 1–3 years (Hammers *et al.*, 2013). Socially dominant individuals are expected to have higher aerobic demands (for territory defence and reproduction) than juveniles, which might explain why haematocrit increases up to 1.5 years-of-age. Interestingly, haematocrit levels at this age (1–3 years) were higher than levels across prime reproductive ages (ca. 4–7 years; Komdeur, 1996; Hammers et al., 2012) which could reflect elevated oxygen demands and/or stress in subordinates competing for dominant social positions at this age (Creel, 2001; Kingma *et al.*, 2016).

We observed a gradual decrease in haematocrit within-individuals with advancing age during most of adulthood. This relationship is similar to that observed in thick-billed murres (*Uria lomvia*) by Elliott et al. (2015), the only other longitudinal study of haematocrit in a wild system to date, and concurs with results from cross-sectional studies. For example, Jégo et al. (2014) found a decrease in haematocrit in roe deer (*Capreolus capreolus*) from the onset of actuarial senescence (8-years old). Thus, declining haematocrit could be indicative of physiological senescence (i.e. diminished

ability to maintain circulating erythrocyte mass) in adult Seychelles warblers. By restricting endurance, such declines could contribute to lower survival in elderly-life; onset of actuarial senescence in the Seychelles warbler is ca. 7 years old (Hammers *et al.*, 2015). However, we did not find evidence of this in our study since only high haematocrit (not low haematocrit) was associated with lower survival, and only in young individuals. Alternatively, decreases in haematocrit could reflect other behavioural and/or physiological changes with age observed in wild vertebrates. For instance, gains in experience could relax demands for oxygen-carrying capacity during foraging (Daunt *et al.*, 2007; Zimmer *et al.*, 2011). Furthermore, the intensity of stress-responses — which can elevate haematocrit (Johnstone et al., 2012) — often decline with age (Wilcoxen *et al.*, 2011; Lendvai *et al.*, 2015). Such changes may be expected of older Seychelles warblers living in long-established territories.

2.5.2. Sex and reproduction

We found that haematocrit was lower in female, compared to male, Seychelles warblers, but only for dominant breeders. This suggests an effect of reproduction on haematocrit, given that dominant breeders produce the vast majority of offspring in the population (Richardson *et al.*, 2001; Raj Pant *et al.*, 2019) and sampling coincided with peaks in breeding activity (Komdeur and Daan, 2005). A well-documented phenomenon (see Fair et al., 2007) in female birds is reproductive anemia – a reduction in haematocrit during egg-laying due to the pleiotropic effects of elevated estrogen (Williams *et al.*, 2004; Wagner *et al.*, 2008). Haematocrit declines observed in other species range from 5–10% (Morton, 1994; Davey et al., 2000) and can persist for several weeks; through incubation and chick-rearing (Williams *et al.*, 2004). In contrast to females, males can have elevated haematocrit prior to and during reproduction as a consequence of elevated testosterone, which stimulates erythropoiesis (Mirand et al., 1965). Therefore, sex-differences may only occur during reproduction (e.g. Morton, 1994). This was not the case in our study since sex-by-status differences were similar both during and outside of breeding attempts. Additionally, there was no evidence of reproductive anaemia (low haematocrit at egg-laying) in dominant females. Taken together, these findings indicate that dominant females maintain hematocrit levels at a constant low-level (relative to dominant males and subordinates of either sex). In other species, estrogen is positively related to territorial behaviors (e.g. singing and aggression) in females (Woodley and Moore, 1999; Pärn *et al.*, 2008). This suggests that female dominance in Seychelles warblers might be accompanied by an upregulation of eostrogen, which subsequently lowers haematocrit. However, we do not currently have data on eostrogen dynamics in this species.

Sex-differences in haematocrit were greatest prior to egg-laying due to increased haematocrit in dominant males. Peak dominant male haematocrit coincided with his female partners fertile period (6 days prior to egg-laying), during which testosterone levels of (pair-bonded) dominant males is

also highest (Van De Crommenacker *et al.*, 2004). Therefore, elevated haematocrit in males may be a consequence of elevated testosterone (e.g. Buttemer and Astheimer, 2000; Ezenwa et al., 2012). A lack of elevated haematocrit in subordinate males supports this explanation, since subordinate males do not elevate testosterone levels during the female fertile period (Van De Crommenacker *et al*., 2004). Elevated haematocrit might also reflect broader behavioural and physiological changes during this period. For example, dominant – but not subordinate – males invest in energetically-costly guarding of mates during their fertile period to prevent extra-pair copulations (Komdeur, 2001). Thus, elevated haematocrit could reflect increased activity levels – and therefore higher oxygen demands – during this critical period for dominant males (see Hammond et al., 2000).

2.5.3. Survival

Intermediate haematocrit levels are predicted to be advantageous for survival, given that both high and low haematocrit are associated with increased mortality in humans and mice (Heller *et al.*, 1998; Wagner *et al.*, 2001; Boffetta *et al.*, 2013). However, we found young Seychelles warblers with low haematocrit had the highest survival. This finding contradicts a study by Bowers et al. (2014) which found that house wren (*Troglodytes aedon*) nestlings with intermediate haematocrit had higher recruitment. However, in this study a more extreme lower range of haematocrit values (i.e. < 30%) was apparent, likely due to age; neonates having lower haematocrit compared to juveniles and adults. Extreme-low haematocrit in neonates likely reflects developmental immaturity, which would reduce the probability of successful fledging (Cornell, Gibson and Williams, 2017).

In adulthood, low haematocrit can be indicative of anaemia (Campbell, 1994), which in wild populations may increase mortality risk via lethargy and fatigue. However, these symptoms are also likely to preclude anaemic individuals from being captured using mist nets. In our sample only 11 individuals were caught with what is considered anaemic haematocrit levels in captive avifauna (<35%; Campbell, 1994). However, the threshold of anaemic haematocrit might be higher in wild populations, given that overall haematocrit can be higher in wild compared to captive populations (Sepp et al., 2010). Nevertheless, the ability to detect a negative survival effect of extreme-low haematocrit in wild populations may be limited by an under-representation of anaemic individuals. Furthermore, haematocrit has been criticised as an indicator of ongoing/recent anaemia due to the disproportionate effect of reticulocytes (O'Brien, et al., 2001; Fair, et al., 2007). These immature erythrocytes are larger and contain less haemoglobin, meaning haematocrit can recover more rapidly than oxygen carrying capacity following anaemic episodes. Therefore, anaemia could impact survival in wild populations without a detectable change in haematocrit values.

Higher haematocrit was associated with reduced survival probabilities in young individuals, despite nearly all haematocrit values falling within what is considered to be a healthy reference range for

captive avifauna; 35–55% (Campbell, 1994). Short-term increases in haematocrit can result from dehydration/haemoconcentration, which in turn limit oxygen carrying capacity, or increase the cardiovascular effort required to maintain optimal oxygen carrying capacity, due to the negative relationship between blood viscosity and flow rate. Several studies have observed a lowering of haematocrit (by ca. 2–5%) in birds during endurance activities via an increase in blood plasma (haemodilution). In line with optimal haematocrit theory (Birchard, 1997), these authors suggest that haemodilution is an adaptive response to prolonged exercise; facilitating faster blood flow for less cardiovascular effort (Jenni *et al.*, 2006; Yap *et al.*, 2018; Bury *et al.*, 2019). Thus, high haematocrit in young Seychelles warblers may reflect a failure to maintain optimal haematocrit, for example, due to dehydration. Alternatively, haematocrit could reflect physiological traits and/or life-histories with potential costs to survival. For example, haematocrit has been positively associated with reproductive effort (e.g. Hõrak, et al., 1998), male ornamentation (Saino et al., 1997), metabolic rate (Yap et al., 2019) and stress (Johnstone et al., 2012). However, further – ideally experiment – studies are needed to confirm the link between haematocrit and pace-of-life in the Seychelles warbler.

2.5.4. Conclusion

Our study provides novel insights into the dynamics of haematocrit, and its impact on survival, in a wild population. Haematocrit was highly variable within individuals and varied in relation to time of day and (in dominant breeders) breeding stage. This variation limits the utility of haematocrit as a marker of age or senescence. However, the overarching relationship observed with advancing age supports the concept of changing oxygen demands with age. Interestingly, we show that haematocrit can be an indicator of survival prospects in wild populations. Whether survival is directly impacted by (suboptimal) oxygen carrying capacity, or factors which increase haematocrit (dehydration, stress etc.) remains to be tested. Since changes in erythrocyte mass occur over longer timescales than, for example, stress hormones and oxidative stress (Bonier *et al.*, 2009; van de Crommenacker *et al.*, 2011, 2017), haematocrit may be a better indicator of an individual's baseline stress-levels (Johnstone et al., 2012). However, short-term changes in blood plasma volume can affect haematocrit levels independently of erythrocyte mass, which makes unravelling the drivers of elevated haematocrit difficult without data on additional blood metrics, such as plasma protein and haemoglobin concentrations (see Johnstone *et al*., 2017). Nevertheless, haematocrit can aid in quantifying physiological state or condition in wild vertebrates, which is a fundamental concept in the study of life-history trade-offs.

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2.7. Supplements

Table S2.1: Haematocrit in relation to cross-sectional age and other factors in Seychelles warblers. Results are from a GAMM analysis with a non-parametric smoothing parameter for age. Significant effects are in bold. The analysis includes only individuals with a known age-at-death; shorter-lived individuals had higher haematocrit.

Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.464	0.006	74.465	< 0.001
Sex (Male)	-0.003	0.003	-0.964	0.335
Status (Dominant)	**-0.019**	**0.004**	**-4.786**	**< 0.001**
Sample Time	**-0.001**	**0.000**	**-3.646**	**< 0.001**
Age at death	**-0.001**	**0.001**	**-2.605**	**0.009**
Sex × Status	**0.023**	**0.004**	**5.231**	**< 0.001**
Smoothed Terms		***df***	**F**	***P***
Age		**7.381**	**13.21**	**<0.001**
Random factors	**854 observations**	**Variance**		
Individual identity	466 individuals	< 0.001		
Breed group	532 breed groups	< 0.001		
Catch year	12 years	< 0.001		

Table S2.2: Haematocrit in relation to cross-sectional age and other factors in Seychelles warblers. Results are from a GAMM analysis with a non-parametric smoothing parameter for age. Significant effects are in bold. The analysis includes only Sexually mature individuals (≥8 months old) caught outside of known breeding attempts.

Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.466	0.008	56.797	< 0.001
Sex (Male)	0.001	0.007	0.206	0.837
Status (Dominant)	**-0.016**	**0.005**	**-2.990**	**0.003**
Sample Time	**-0.002**	**0.001**	**-3.865**	**< 0.001**
Sex × Status	**0.020**	**0.008**	**2.511**	**0.012**
Smoothed Terms		***df***	**F**	***P***
Age		**1.885**	**5.874**	**0.017**
Random factors	**374 observations**	**Variance**		
Individual identity	297 individuals	< 0.001		
Breed group	272 breed groups	< 0.001		
Catch year	13 years	< 0.001		

Table S2.3: Haematocrit in relation to cross-sectional age and other factors in Seychelles warblers. Results are from a GAMM analysis with a non-parametric smoothing parameter for age and time to lay date (days). Significant effects are in bold. The analysis includes only dominant breeder individuals caught within 50 days of known breeding attempts.

Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.437	0.007	65.409	< 0.001
Sex (Male)	**0.028**	**0.003**	**10.065**	**< 0.001**
Sample Time	**-0.001**	**0.000**	**-2.694**	**0.007**
Smoothed Terms		***df***	**F**	***P***
Age		**1.674**	**3.102**	**0.035**
Time to lay date (Female)		**4.05**	**3.211**	**0.014**
Time to lay date (Male)		**5.144**	**5.313**	**< 0.001**
Random factors	**436 observations**	**Variance**		
Individual identity	281 individuals	< 0.001		
Breed group	322 breed groups	< 0.001		
Catch year	12 years	< 0.001		

Table S2.4: Haematocrit in relation to cross-sectional age and other factors in Seychelles warblers. Results are from a GAMM analysis with a non-parametric smoothing parameter for age and time to lay date (days). Significant effects are in bold. The analysis includes only sexually mature (≥ 8 months old) subordinate individuals caught within 50 days of known breeding attempts (of the subordinates breed group).

Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.468	0.012	40.568	< 0.001
Sex (Male)	0.007	0.005	1.223	0.223
Sample Time	**-0.002**	**0.001**	**-2.527**	**0.012**
Smoothed Terms		***df***	**F**	***P***
Age		1.823	2.174	0.209
Time to lay date (Female)		**2.253**	**3.445**	**0.032**
Time to lay date (Male)		1	0.986	0.322
Random factors	**182 observations**	**Variance**		
Individual identity	140 individuals	< 0.001		
Breed group	140 breed groups	< 0.001		
Catch year	12 years	< 0.001		

Table S2.5: Haematocrit in relation to cross-sectional age (Mean age) and longitudinal age (Δ Age) in Seychelles warbler <1.5 years old and 1.5–13 years old. Parameters shown are from LMM analysis. Significant effects are in bold. Model excludes dominant breeding individuals caught during key breeding stages, where haematocrit deviates from typical levels; 20 days before to 5 days after laying for dominant males, and 30 – 50 days after laying in dominant females.

< 1.5 YEARS OLD				
Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.392	0.010	40.682	< 0.001
Mean Age	**0.160**	**0.022**	**7.351**	**< 0.001**
Mean Age2	**0.037**	**0.008**	**4.533**	**< 0.001**
Δ Age	**-0.086**	**0.016**	**-5.457**	**< 0.001**
Δ Age2	**-0.052**	**0.018**	**-2.968**	**0.003**
Sex (Male)	-0.005	0.003	-1.579	0.115
Status (Dominant)	**-0.014**	**0.007**	**-2.08**	**0.038**
Sample Time	**-0.002**	**0.000**	**-3.272**	**0.001**
Sex × Status	0.015	0.008	1.75	0.081
Random factors	**625 observations**	**Variance**		
Individual identity	499 individuals	< 0.001		
Breed group	448 breed groups	< 0.001		
Catch year	14 years	< 0.001		
1.5 - 13 YEARS OLD				
Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.463	0.007	70.789	< 0.001
Mean Age	**-0.002**	**0.001**	**-2.899**	**0.004**
Δ Age	**-0.002**	**0.001**	**-2.728**	**0.007**
Sex (Male)	**0.021**	**0.003**	**7.783**	**< 0.001**
Status (Dominant)	**-0.013**	**0.004**	**-3.539**	**< 0.001**
Sample Time	**-0.002**	**0.000**	**-3.681**	**< 0.001**
Random factors	**625 observations**	**Variance**		
Individual identity	372 individuals	< 0.001		
Breed group	469 breed groups	< 0.001		
Catch year	14 years	< 0.001		

Table S2.6; Survival in the Seychelles warbler in relation to haematocrit for individuals 1.5 years old and 1.5–13 years old. Results are from binominal GLMMs with survival to the following year (Y/N) as the response variable. Significant effects are in bold. Model excludes dominant breeding individuals caught during key breeding stages, where haematocrit deviates from typical levels; 20 days before to 5 days after laying for dominant males, and 30 – 50 days after laying in dominant females.

< 1.5 YEARS OLD				
Predictor	**β**	**SE**	***z***	***P***
(Intercept)	5.114	1.284	3.981	< 0.001
Haematocrit	**-9.565**	**3.009**	**-3.179**	**0.001**
Sex (Male)	-0.236	0.223	-1.057	0.291
Status (Dominant)	0.394	0.376	1.048	0.295
Age	0.502	0.397	1.264	0.206
Random factors	**497 observations**	**Variance**		
Breed group	414 breed groups	< 0.001		
Catch year	14 years	0.327		
1.5 - 13 YEARS OLD				
Predictor	**β**	**SE**	***z***	***P***
(Intercept)	1.688	1.816	0.929	0.353
Haematocrit	-1.692	4.012	-0.422	0.673
Sex (Male)	-0.489	0.267	-1.833	0.067
Status (Dominant)	**0.707**	**0.349**	**2.024**	**0.043**
Age	-0.028	0.045	-0.634	0.526
Random factors	**348 observations**	**Variance**		
Catch year	13 years	0.238		

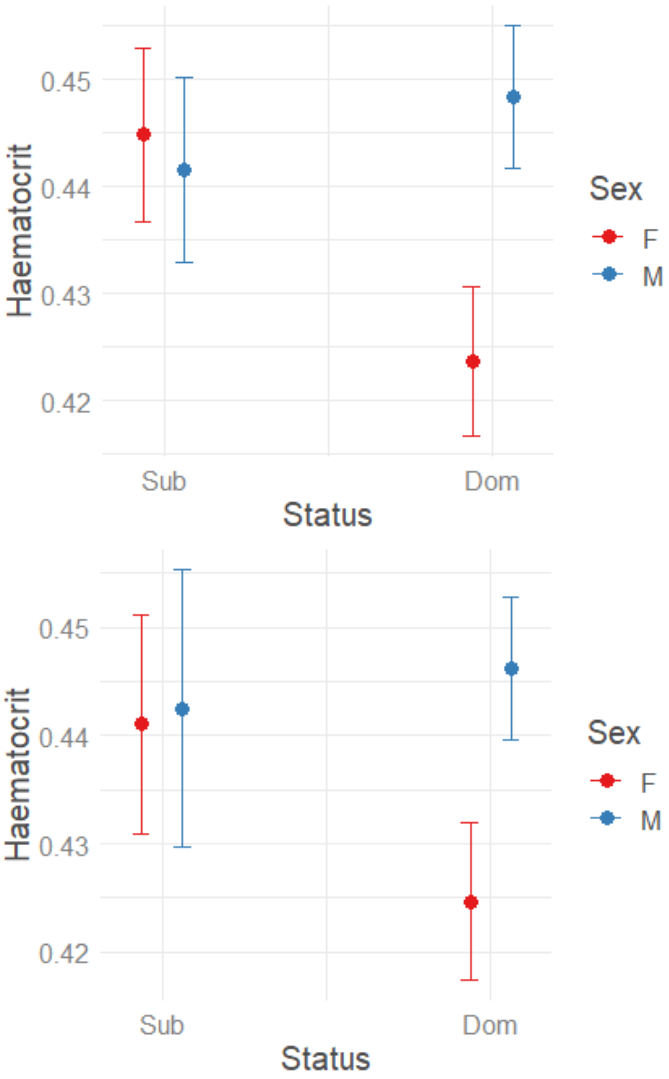

Figure S2.1; Haematocrit in relation to sex and status of Seychelles warblers. Points are model predicted haematocrit ± 95% CI, adjusted for age and time of sampling (held at sample average). The top plot includes all individuals of all ages, irrespective of breeding stage (*N* = 1379). The bottom plot only includes sexually mature (≥8 months old) individuals caught outside of known breeding attempts (*N* = 374). Sub = Subordinate, Dom = Dominant breeder.

Figure S2.2; Model predicted haematocrit in relation to age in Seychelles warblers. Plots are derived from models summarised in tables S2.1, S2.2 and S2.3. Datasets include; A) All individuals that died before the time of analysis (controlling for age-at-death); B) All sexually-mature (> 8 months-old) sampled outside of known breeding attempts; c) All dominant individuals sampled during breeding attempts (controlling for days from lay date).

Chapter 3
Causes and Consequences of Telomere Lengthening in a Wild Vertebrate Population

Beachside hammock → stress-free warbler → telomere lengthening

3.1. Abstract

Telomeres have been advocated to be important markers of biological age in evolutionary and ecological studies. Telomeres usually shorten with age and shortening is frequently associated with environmental stressors and increased subsequent mortality. Telomere lengthening – an apparent increase in telomere length between repeated samples from the same individual – also occurs. However, the exact circumstances, and consequences, of telomere lengthening are poorly understood. Using longitudinal data from the Seychelles warbler (*Acrocephalus sechellensis*), we tested whether telomere lengthening – which occurs in adults of this species – is associated with specific stressors (reproductive effort, food availability, malarial infection and cooperative breeding) and predicts subsequent survival. In females, telomere shortening was observed under greater stress (i.e. low food availability, malaria infection), while telomere lengthening was observed in females experiencing lower stress (i.e. high food availability, assisted by helpers, without malaria). The telomere dynamics of males were not associated with the key stressors tested. These results indicate that, at least for females, telomere lengthening occurs in circumstances more conducive to self-maintenance. Importantly, both females and males with lengthened telomeres had improved subsequent survival relative to individuals that displayed unchanged, or shortened, telomeres – indicating that telomere lengthening is associated with individual fitness. These results demonstrate that telomere dynamics are bidirectionally responsive to the level of stress that an individual faces, but may poorly reflect the accumulation of stress over the lifetime.

3.2. Introduction

Senescence – the deterioration of health and performance in old age – occurs in nearly all species (Nussey *et al.*, 2013). However, within species there can be considerable individual variation in the onset and rate of senescence (e.g. Lemaître *et al.*, 2013). Thus, individuals may be biologically older or younger than expected for their chronological age. Measuring 'biological age' (Baker and Sprott, 1988) is valuable, not only in regards to organismal health but also in terms of understanding fundamental concepts in ecology and evolution e.g. trade-offs in life-history strategies, or the impact of different environmental stressors (Stearns, 2008; Lemaître *et al.*, 2015).

Telomeres are repetitive nucleotide sequences at the ends of chromosomes, which protect the functional integrity of the genome. Due to the 'end replication problem' (Watson, 1972), telomeres shorten with each cell division, until a critical length is reached where cells can no longer divide (Olovnikov, 1996; Campisi, 2003) Telomeres also shorten when exposed to sources of cellular damage, such as reactive oxygen species (Von Zglinicki, 2002; Reichert and Stier, 2017). Both the number of cell-divisions and cell damage load are cumulative (i.e. age-dependent) but also variable

in rate. Telomere length shortens with increasing age in a broad range of taxa (Barrett *et al.*, 2013; Bendix *et al.*, 2014; Stier *et al.*, 2015) but see Fairlie *et al.* (2016).

There is considerable empirical evidence to support the idea of telomere length, and the rate of shortening, being a marker of biological age. Accelerated telomere shortening occurs as an outcome of life-history or environmental conditions associated with increased cellular division and reactive oxygen species production, including developmental growth (Salomons *et al.*, 2009; Monaghan and Ozanne, 2018), early-life adversity (Boonekamp *et al.*, 2014; Watson, Bolton and Monaghan, 2015), reproductive effort (Reichert *et al.*, 2014; Sudyka *et al.*, 2014) and stress (Chatelain, Drobniak and Szulkin, 2020). Shorter telomeres and/or higher attrition rates are associated with increased mortality risk (e.g. Haussmann, Winkler and Vleck, 2005; Vera *et al.*, 2012; Fairlie *et al.*, 2016; Barrett *et al*., 2013) and 'faster' life histories (Haussmann *et al.*, 2003; Sudyka, Arct, *et al.*, 2019). Therefore, telomere length has been proposed as a valuable biomarker linking past life-history costs to future performance (Young, 2018). Additionally, telomeres can reflect an individual's ability to cope with stresses that would otherwise shorten telomeres. For example, individuals with higher reproductive success often have longer telomeres, despite the stress associated with reproduction (Bauch, Becker and Verhulst, 2012; Angelier *et al.*, 2019; Sudyka, 2019). Therefore, as well as reflecting past stress and life-history costs, telomere length has been proposed as a marker of intrinsic quality (Pauliny *et al.*, 2006; Angelier *et al.*, 2019).

Over the last decade there has been a rapid expansion in studies investigating the causes and consequences of telomere dynamics across a wide range of taxa and environmental situations. However, our growing awareness of the complexity of telomere dynamics raises important questions on how we interpret telomeres as a biomarker. Importantly, multiple longitudinal studies in humans and some wild vertebrates have shown that within-individual changes in telomere length are highly variable (Svenson *et al.*, 2011; Fairlie *et al.*, 2016; Hoelzl *et al.*, 2016; Spurgin *et al.*, 2017; van Lieshout *et al.*, 2019). Thus, authors have questioned whether single measures of telomere length per individual can accurately reflect biological age and/or individual quality; indeed, several studies have shown the rate of within-individual change in telomere length, rather than absolute telomere length, is more informative of past life-history costs and future performance (Salomons *et al.*, 2009; Boonekamp *et al.*, 2014; Wood and Young, 2019). Furthermore, the telomere dynamics observed in longitudinal studies (see above) are frequently bidirectional, i.e. an individual's telomeres may lengthen as well as shorten. Until recently, observed telomere lengthening was often attributed to measurement error between samples collected too close in time – relative to the rate of telomere loss – to detect telomere shortening (Chen *et al.*, 2011; Steenstrup *et al.*, 2013). However, it is now recognised that the degree or frequency of observed telomere lengthening is often greater than that expected from measurement error alone (Bateson and Nettle, 2017; Spurgin *et al.*, 2017; van Lieshout *et al.*, 2019).

Telomere lengthening within the same individual may be observed for a variety of reasons. First, the enzyme telomerase can restore lost telomere length (Blackburn *et al.*, 1989). Since telomeres shorten during cell division, telomerase remains active in cell lineages requiring greater proliferation potential, such as haematopoietic stem cells (Morrison *et al.*, 1996; Haussmann *et al.*, 2007). Telomeres can also lengthen via alternative mechanisms, independent of telomerase (see Cesare and Reddel, 2010 for a discussion). Importantly, telomere measurements may increase in subsequent assays due to changes in clonal cell composition, i.e. an increase in long-telomere cells relative to short-telomere cells. All the mechanisms explained above are relevant to the telomere dynamics of blood, the tissue most often utilised for ecological and evolutionary studies on vertebrates (Nussey *et al.*, 2014). Furthermore, in mammals the proportions of circulating leucocyte cell types (with differing telomere lengths; Weng, 2012) can also change dramatically within an individual, for example in response to infection, resulting in apparent changes in overall telomere length (Beirne *et al.*, 2014). In birds and reptiles, blood-derived assays of telomere length overwhelmingly stem from nucleated erythrocytes (Stier *et al.*, 2015), and telomerase activation or turnover in haematopoietic cell lines could, in theory, create heterogeneity in measured telomere length.

The importance of telomere lengthening in wild populations remains uncertain. Since telomere attrition occurs as a consequence of life-history or environmental stress costs, telomere lengthening may reflect investment in self-maintenance when those costs are alleviated. For example, wild edible dormice (*Glis glis*) that receive supplementary food showed lengthened telomeres (Hoelzl *et al.*, 2016). In other wild species, changes in telomere length reflected temporal differences in environmental conditions, with lengthening coinciding with more favourable environments (e.g. Mizutani *et al.*, 2013; Foley *et al.*, 2020). Telomere dynamics can also reflect changes in parasitic pressure. For example, infection with malaria has been associated with telomere attrition in wild and captive birds (Asghar *et al.*, 2016), but the clearing of infections in humans can result in lengthening (Asghar *et al.*, 2018). The ability of telomeres to both shorten and lengthen, rather than being an irreversible one-way ratchet, suggests that we may have to rethink our interpretation of telomere dynamics. Instead of reflecting the accumulation of all past stressors and growth, telomere length may be more of a short-term marker, reflecting an individual's current condition consequent on the challenges and trade-offs faced by an individual. However, in contrast to telomere shortening, the circumstances under which telomere lengthening occurs in natural populations remain poorly understood.

Given the fitness costs associated with shorter, or more rapidly shortening, telomeres (see above), one might expect improved fitness to be associated with telomere lengthening. Recent reviews argue that telomere dynamics are a non-causal biomarker of accumulated cellular damage – such as that occurring from oxidative stress – that subsequently impacts fitness (Simons, 2015; Young,

2018). However, there is evidence that active restoration of telomere length can impact organismal performance. First, telomere lengthening could reduce the frequency of critically short telomeres – thought to directly contribute to organismal ageing by inducing cellular senescence and apoptosis (Vera *et al.*, 2012; Van Deursen, 2014). Secondly, telomerase has restorative effects on cells (Cong and Shay, 2008; Criscuolo *et al.*, 2018). Both telomerase activity and telomere lengthening are associated with tissue regeneration (Anchelin *et al.*, 2011; Reichert, Bize, *et al.*, 2014) and telomerase overexpression in mice is beneficial to a range of health parameters (Bernardes de Jesus *et al.*, 2012; Simons, 2015). Conversely, active telomere lengthening could also have negative effects, such as proliferating cancers (Shay and Wright, 2011) or by diverting energy from competing traits (Young, 2018). Nonetheless, telomere lengthening has the potential to be associated with organismal performance, and this impact is not dependent on telomere length playing a causal role in organismal ageing.

In this study, we aim to determine when and why telomere lengthening occurs, and assess its association with survival, in a wild population of the facultative cooperatively breeding Seychelles warbler (*Acrocephalus sechellensis*). Previous studies on this population have shown that telomeres shorten with age, and individuals with shorter telomeres are less likely to survive to the following year (Barrett *et al.*, 2013). Furthermore, telomere shortening is associated with various stresses in this species, including inbreeding (Bebbington *et al.*, 2016), intra-specific antagonistic interactions (Bebbington *et al.*, 2017) and parental care (Hammers *et al.*, 2019). However, telomere lengthening occurs frequently (i.e. 44% of successive samples taken from the same adult individual exhibit an increase in telomere length), and is greater than that expected from measurement error alone (Spurgin *et al.*, 2018). We predict that telomere lengthening occurs in individuals that experience reduced stress. Specifically, we predict that, for adults, telomere lengthening will be associated with reduced reproductive effort (less breeding, higher food availability and the presence of helpers) and an absence of malaria (the only known parasite in the population). We expect this relationship to be sex-specific, given that reproductive investments differ between sexes in this species; females lay the eggs, undertake incubation and have higher nestling provisioning rates than do males (Hammers *et al.*, 2019; van Boheemen *et al.*, 2019). Furthermore, we tested whether telomere lengthening is associated with increased survival.

3.3. Methods

3.3.1. The Seychelles warbler model system

The Seychelles warbler is a small insectivorous passerine currently distributed across five islands in the Seychelles. The population on Cousin Island (29 ha; 4°20’ S, 55°40’ E) – containing *ca.* 320 individuals – has been extensively monitored since 1986 (Komdeur, 1992; Hammers *et al.*, 2015).

Since 1997, nearly all individuals (>96%) have been ringed with a unique combination of a British Trust for Ornithology (BTO) metal ring and three colour rings for identification (Richardson *et al.*, 2001; Raj Pant *et al.*, 2020). Individuals were usually first caught as nestlings, or as dependent juveniles (<8 months old) with mist nets (see Kingma *et al.*, 2016 for details). Juveniles were aged as fledglings (1–3 months), old fledglings (3–5 months) or sub-adults (5–12 months) based on behaviour and eye colour (Komdeur, 1992). Since the resighting probability of individuals during the major breeding season is close to one – 0.98 for individuals ≥2 years-old (Brouwer *et al.*, 2006) – and dispersal from the island is virtually absent (Komdeur *et al.*, 2004), individuals that were not observed during the major breeding season were assumed dead. First year survival is 0.61 ± 0.09 SE, increasing to 0.84 ± 0.04 SE annual survival in adults (Brouwer *et al.*, 2006). For individuals reaching fledgling age, the mean life expectancy is 5.5 years (Komdeur, 1991), and the maximum recorded lifespan is 19 years (Hammers and Brouwer, 2017).

The population is structured into *ca* 115 clearly defined territories (Kingma *et al.*, 2016). The availability of the warbler's invertebrate prey (Komdeur, 1992) varies considerably due to the interacting effects of defoliating salt spray (along coastal territories), tree species abundance, elevation and rainfall (Van de Crommenacker *et al.*, 2011). Territories are defended year-round by a single dominant breeding pair, but *ca* 40% of territories include an additional 1–5 sexually mature subordinates, often past offspring of the same dominant pair (Richardson, Burke and Komdeur, 2002). The majority of breeding activity (94% of territories) occurs from June to August, but a minor breeding season also occurs from January to March (Komdeur and Daan, 2005). Breeding attempts usually consist of one-egg clutches (Komdeur, 1994). Only females incubate while both sexes provision chicks and fledglings for *ca* three months post-fledging. Around one third of subordinates also provide alloparental care to group offspring, hereafter 'helpers' (Komdeur, 1994; Hammers *et al.*, 2019). About 44% of female helpers are also co-breeders (Richardson *et al.*, 2001; Raj Pant *et al.*, 2019). The offspring of cobreeders are jointly cared for by the subordinate female and dominant pair (Richardson *et al.*, 2001; Bebbington *et al.*, 2018). The frequency of extra-pair paternity in the population is high (*ca* 41%; Raj Pant *et al.*, 2019) and such paternity is nearly always gained by dominant males from other territories (Richardson *et al.*, 2001), but males only provide parental care in their own territory.

3.3.2. Data collection

Our study uses data collected from 1995 to 2015. Each year (June–September), during the major breeding season, each territory was visited at least every two weeks to determine the identity and status of group individuals. During visits, the dominant female was followed for at least 15 minutes to assess breeding activity (Richardson, Burke and Komdeur, 2007). Territories with an active nest

were visited every 3–4 days until the nestling(s) have fledged or the breeding attempt failed. Observations of incubating and/or provisioning were used to estimate hatching/fledging dates, and to determine whether any subordinates present in the territory were helpers (Richardson, *et al*., 2002; van Boheemen *et al.*, 2019). For each territory, the availability of food was calculated (following Komdeur, 1992). Briefly, the number of insects (on the undersides of leaves) was multiplied by the percentage cover of broad-leaf vegetation within territories. This number was then divided by the number of adult territory occupants to give food availability per individual (Brouwer *et al.*, 2006).

During each major breeding season, as much of the adult population as possible (normally around 30%) was caught and re-sampled: *ca* 25 ul of blood was taken from the brachial vein and stored in 100% ethanol (Richardson *et al.*, 2001). DNA extracted from the blood samples (following Richardson *et al.*, 2001) was used to confirm sex and assign parentage using MasterBayes 2.52 based on genotypes derived from 30 microsatellite loci (for details see Sparks *et al.*, 2020). The presence of haemosporidian infection (*Haemoproteus nucleocondensus*; hereafter referred to as malaria) - the only known parasite in the Seychelles warbler (Hutchings, 2009) - was screened for following Hellgren *et al*. (2004). In the Seychelles warbler, nearly all individuals (ca. 85%) become infected with malaria in their first year (Hammers *et al.*, 2016). In adults, the prevalence of malaria is much lower (ca. 20% at 4 years-of-age) as individuals clear the initial infection, or enter the latent infection stage (where parasites may be absent in blood - and hence are not detected - but persist at low abundance in internal organs. Thus, in our study (of individuals >1 year of age), individuals in which we detected malaria (hereafter "infected") are either in the late chronic stage of their initial infection, or in subsequent relapses, or reinfections (Valkiūnas, 2005). Infection with malaria does not appear to have an impact on annual survival in the Seychelles warbler (Hammers *et al.*, 2016) but has been linked to telomere attrition in the great reed warbler (*Acrocephalus arundinaceus*: Asghar *et al.*, 2015).

3.3.3. Telomere analysis

Relative Telomere Length (RTL; the concentration of amplified telomeric DNA relative to that amplified at *GAPDH* – a single copy gene) had previously been measured using qPCR as part of another study (Spurgin *et al.*, 2018). Intra-plate repeatability of *GAPDH* and Telomere Cq values are 0.74 (CI = 0.74, 0.75) and 0.73 (CI = 0.71, 0.74), respectively. Inter-plate repeatability of RTL is 0.68 (CI = 0.65, 0.70); based on 422 samples measured at least twice at different time points. The within-individual variance of RTL (i.e. from multiple samples across an individual's life-time) is greater than the variance among repeated measurements of the same sample (Levene's test: $F = 43.63$; $p < .001$). Importantly, this is true when incidences of within-individual decreases and increases in RTL (i.e. between successive samples from the same individual) and analyzed separately, indicating that the

magnitude of both telomere shortening and (crucial) lengthening observed in our system is greater than that expected from measurement error alone (Spurgin *et al.*, 2018). Since avian erythrocytes are nucleated and vastly outnumber other blood cell types, blood RTL is effectively a measure of erythrocyte RTL (Stier *et al.*, 2015). Individuals with two or more RTL measurements were used in the current study, with the difference between consecutive pairs of RTL measurements (∆RTL) as the response variable. We excluded RTL measurements from young individuals (< 1 year), as previous work in this population has shown that within-individual rate of attrition per annum is an order of magnitude greater in the first year compared to adult life (Spurgin *et al.*, 2018). For consistency, we focused on RTL measurements from catches only within the major breeding season, since inter-seasonal ΔRTL could reflect seasonal effects on RTL. Individuals are caught opportunistically, meaning that the follow-up duration between RTL measurements (hereafter ΔRTL period) ranged from one year (i.e. consecutive seasons) to 9 years (Fig. S3.1). ΔRTL is not associated with the duration of the ΔRTL period. The final dataset comprised 359 ∆RTL measures from 227 adults.

Reproductive effort was measured as the number of offspring raised by an individual in the ΔRTL period; specifically, offspring that had hatched after time 1 and had reached independence (3 months old) before time 2. Social offspring – those for which a dominant breeder provides parental care – are determined from behavioural observations during nest attempts. Offspring are genotyped to identify genetic parentage. This is an underestimation of total offspring produced, since we excluded offspring for which parents could not be assigned (*ca* 15% of offspring) and some offspring are likely to have died before being sampled (Edwards, Burke and Dugdale, 2017). We used the number of social offspring as our estimate of reproductive output in males (since males do not care for offspring sired in other territories). Females (dominants or co-breeder) always contribute to the care of any offspring in the nest; (Richardson, Burke and Komdeur, 2002). Thus, female reproductive output was the number of co-bred offspring. For both sexes, the majority of individuals had 0–2 offspring within each ΔRTL period (Fig. S3.1). Offspring number was positively correlated with the ΔRTL period (Pearson's $r = 0.69$, $df = 357$, $P < 0.001$, Fig. S3.1), meaning that individuals typically produced one offspring every two years.

During the ΔRTL period, we averaged food availability (insect abundance per individual per field season) across field seasons. Reproductive effort - in terms of time spent incubating and provisioning - of dominant breeders is reduced by the presence of helpers, including co-breeders (Hammers *et al.*, 2019; van Boheemen *et al.*, 2019). Reduced telomere attrition in dominant females has been associated with the presence of helpers in a previous study (Hammers *et al.*, 2019). Therefore, we determined whether nest helpers (including co-breeders) were present in the territories of individuals that produced offspring.

3.3.4. Statistical analysis

Using RStudio (version 1.2.5033 and R version 4.0.3, Rstudio Team 2020) we tested whether food availability, number of offspring, helper presence and malaria status predicted ΔRTL, with the prediction that high food availability, low reproductive output, helper presence and no malaria infection - or a combination of these factors - would result in telomere lengthening. We adjusted ΔRTL following Verhulst *et al.* (2013a); this method subtracts the mean difference between successive samples expected from the regression-to-mean effect, estimated by the correlation between successive samples. In our dataset, this correlation was very weak (Pearson's $r = 0.06$, $df = 357$ $P = 0.22$), as expected given the low within-individual consistency of RTL in this system (Spurgin *et al.*, 2018). This results in an adjusted ΔRTL (hereafter ΔRTL) which is equivalent to RTL at time 2; positive values indicate longer RTL and negative values indicate shorter RTL, relative to the population mean RTL. As expected, ΔRTL was strongly correlated with unadjusted ΔRTL (Pearson's $r = 0.71$, $df = 357$, $P < 0.001$), meaning that individuals with lengthened or shortened RTL (relative to their initial RTL) tended to have more positive or more negative ΔRTL, respectively.

The association between factors and ΔRTL was tested using Linear Mixed Models (package *lme4* v1.1-25; Bates *et al.*, 2015). We deliberately focused on a restricted set of fixed effects – chosen a priori based on logic and evidence of influencing telomere dynamics – to avoid data dredging, which could generate false-positive associations. Chosen fixed effects during the RTL period included; mean food availability (continuous), number of offspring (continuous), the duration of the RTL period (continuous) and the presence of helpers (yes or no). Chosen fixed effects at the start of the RTL period included; age (continuous) and malaria status (infected or uninfected). We also included logical two-way interactions; for example, the effect of offspring production on telomere maintenance may depend on food availability and/or helper presence. These effects were likely to differ between the sexes due to differing investments in reproduction (see above). Indeed, initial models (that included both males and females) indicated that associations between ΔRTL and fixed effects depended on two- and three-way interactions with sex (Table S3.1). To investigate these sex-specific differences in more detail, while also avoiding the need for multiple three-way interactions, separate models were created for males and females. Offspring number, food availability and ΔRTL period were log10 transformed (for normality) and mean-centered to remove collinearity between their main effects and interaction (Schielzeth, 2010). All main fixed effects were kept in final models (regardless of significance) but two-way interactions were removed when non-significant (see Whittingham et al., 2006). Since most individuals had 0–2 offspring, offspring number was reduced to a categorical variable (zero, one or ≥2) for graphical interpretation of interactions.

We included catch year of the first RTL measurement as a random factor, since the subsequent change in RTL may depend on year-to-year effects. Cohort year was not included as a random factor since Spurgin et al. (2018) previously found no support for cohort-effects influencing ΔRTL. Variation in RTL between qPCR plates (Sparks *et al.*, 2020) could contribute to variation in ΔRTL, since RTL measurements from longitudinal samples were run on separate plates. Therefore, the plate identities of both RTL measurements per ΔRTL were included as random factors. Individuals for which >2 RTL measurements were available had multiple measures of ΔRTL. For example, an individual with three RTL measurements would have two measures of ΔRTL; between measurements 1–2 and 2–3. ΔRTL is previously shown to be highly variable across the same individual's life-time (i.e. Individuals do not exhibit consistent lengthening or shortening; Spurgin et al. 2018). Thus, in our analysis we used all available measures of ΔRTL per individual and included individual identity as a random factor. Our findings were consistent when only one datum per individual was used.

To test whether ΔRTL influenced subsequent survival, we performed a Cox proportional hazards regression analysis (package *survival* v3.2-7; Therneau, 2014). The response variable was the number of years an individual lived beyond the sampling date of its last RTL measurement. We included 23 individuals that were still alive in 2020 as right-censored data points. 13 individuals translocated to other islands post-sampling were excluded, leaving 214 individuals. Predictor variables were ΔRTL, sex and age at the last RTL measurement (since older individuals are expected to have shorter remaining lifespans). Where multiple measures of ΔRTL were available per individual, we used only the last measurement, meaning that each ΔRTL value represents the last known change in telomere length before the individual died. We were interested in whether predicted hazard ratios exhibited a proportional change across the range of ΔRTL values, or whether the association was nonlinear. Therefore, we modelled ΔRTL as both a linear and quadratic function. In this model, positive hazard coefficients would indicate a decreased probability of survival with increasing values of the predictor variable. Hazard ratios represent the effect size of predictors; for example, a hazard ratio of 2 indicates that the risk of death is twice as high for the corresponding change in a predictor variable.

3.4. Results

3.4.1. Telomere dynamics

Out of the 359 pairs of consecutive RTL measurements included in our analysis, 166 (46%) showed an increase in length. There was no difference in ΔRTL between males and females (Mean ± SE; Males = 0.001 ± 0.014, Females = 0.000 ± 0.013). Females infected with malaria at first RTL measurement had more negative ΔRTL than non-infected females (Fig. 3.1). In females, the association between offspring number and ΔRTL was dependent on the availability of food within

the same period (interaction term in Table 3.1). Females that produced fewer offspring had ΔRTL close to zero regardless of food availability (Fig. 3.2; females with zero and one offspring). Females in territories of low mean food availability had a more negative ΔRTL with increasing numbers of offspring, whereas females in territories of high mean food availability had a more positive ΔRTL with increasing numbers of offspring (Fig. 3.2). Females that reared offspring with the assistance of helpers also had more positive ΔRTL change compared to females without helpers (Fig. 3.3). ΔRTL was not associated with ΔRTL period or age (Table 3.1). None of the chosen explanatory variables predicted ΔRTL in males (Table 3.1). In all models, results were qualitatively identical when unadjusted ΔRTL was used as the response variable and controlling for initial RTL (Table S3.2).

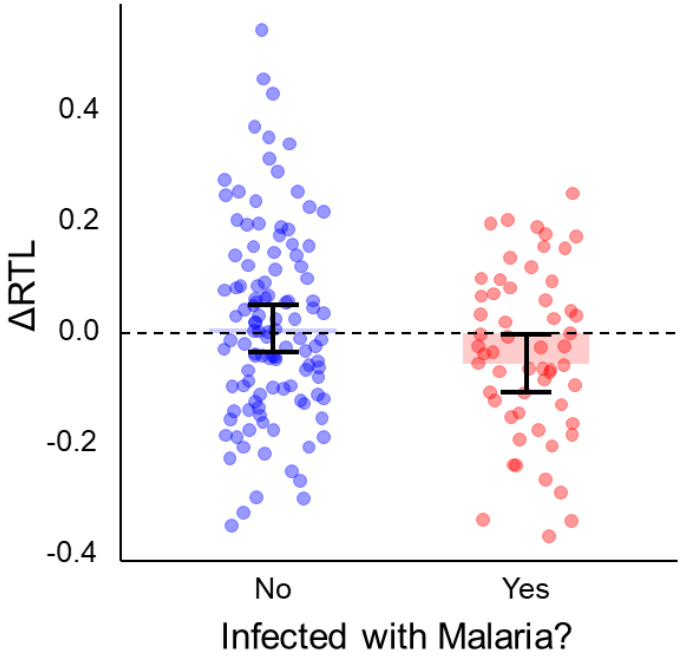

Figure 3.1: The relationship between within-individual change in RTL and malaria status (at initial RTL measurement) for female Seychelles warblers. Negative values of ΔRTL indicate telomere shortening, while positive values indicate telomere lengthening. Bars are model predicted values (Table 1) of ΔRTL with 95% CI for individuals with and without malaria, adjusted for offspring number, food availability and helper status (reference category = no helper). Points are raw data.

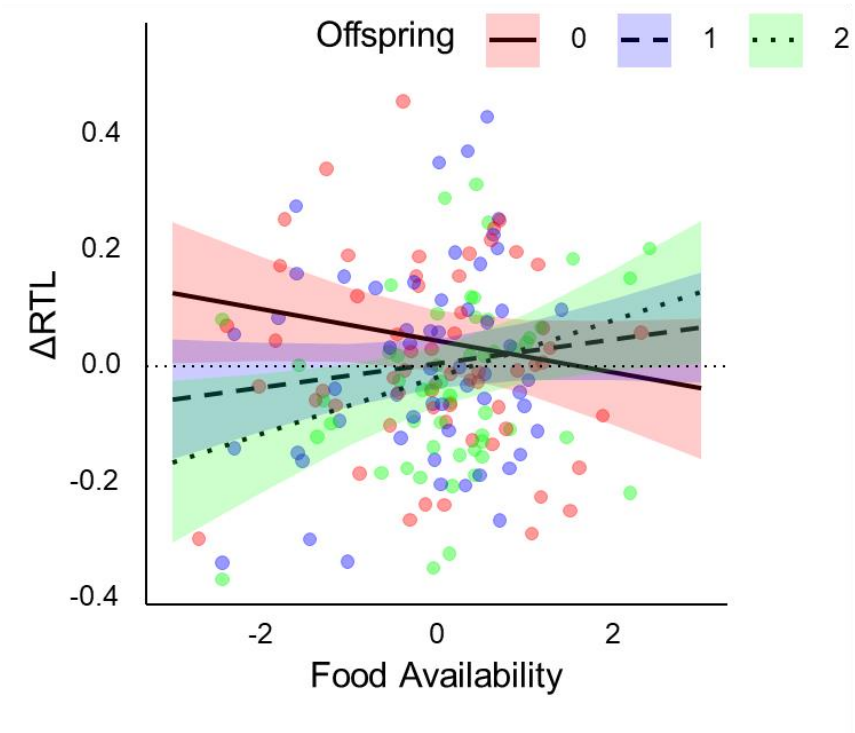

Figure 3.2. The relationship between within-individual change in RTL, mean food availability (mean annual insect-prey availability) and offspring production for female Seychelles warblers. Negative values of ΔRTL indicate telomere shortening, while positive values indicate telomere lengthening. Lines are model predicted slopes (Table 1) with 95% CI for individuals with none, one and two offspring, adjusted for malaria status (reference category = uninfected) and helper presence (reference category = no helper). Points are raw data grouped by number of offspring (zero, one or ≥ two).

Table 3.1: Linear mixed effects models explaining variation in ΔRTL between consecutive blood samples from the major breeding seasons in female and male Seychelles warblers. Brackets include the reference category for binomial terms.

FEMALES					
Predictor	**β**	**SE**	***df***	***t***	***P***
(Intercept)	0.036	0.032	92.293	1.119	0.266
Food availability	0.017	0.015	82.367	1.127	0.263
Number of offspring	**-0.033**	**0.016**	154.301	**-2.096**	**0.038**
Helper presence (Yes)	**0.080**	**0.037**	150.072	**2.163**	**0.032**
Malaria (Yes)	**-0.064**	**0.027**	157.627	**-2.322**	**0.022**
ΔRTL period	-0.002	0.017	138.682	-0.126	0.900
Age	-0.007	0.006	155.293	-1.261	0.209
Food × Offspring	**0.040**	**0.014**	158.938	**2.893**	**0.004**
Random factors	**170 observations**	**Variance**			
Individual identity	108 individuals	< 0.001			
Catch year	18 years	0.001			
qPCR plate identity 1	54 plates	0.001			
qPCR plate identity 2	49 plates	0.002			
Residual		0.021			
MALES					
Predictor	**β**	**SE**	***df***	***t***	***P***
(Intercept)	0.004	0.025	33.224	0.152	0.880
Food availability	-0.002	0.015	95.464	-0.113	0.910
Number of offspring	0.001	0.018	163.069	0.074	0.941
Helper presence (Yes)	0.016	0.039	173.784	0.394	0.694
Malaria (Yes)	-0.008	0.030	164.176	-0.286	0.775
ΔRTL period	-0.019	0.018	113.981	-1.035	0.303
Age	-0.015	0.015	166.929	-1.035	0.302
Random factors	**189 observations**	**Variance**			
Individual identity	119 individuals	0.006			
Catch year	16 years	0.001			
qPCR plate identity 1	65 plates	0.001			
qPCR plate identity 2	56 plates	0.005			
Residual		0.026			

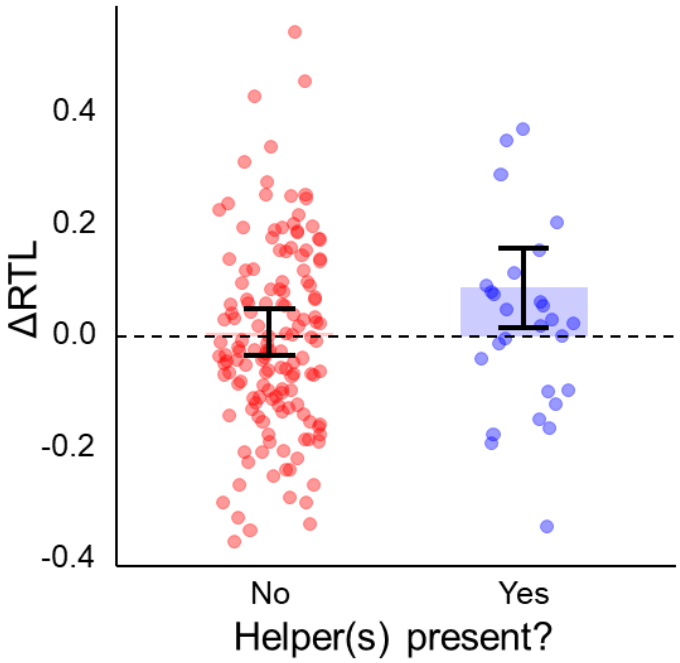

Figure 3.3: The relationship between within-individual change in RTL and helper presence for female Seychelles warblers. The 'Yes' group contains females that had one or more helpers (or co-breeders) assisting with the care of at least one offspring during the ΔRTL period (time between samples). Negative values of ΔRTL indicate telomere shortening, while positive values indicate telomere lengthening. Bars are model predicted values (Table 1) with 95% CI for individuals with and without helpers, adjusted for offspring number and food availability and malaria status (reference category = uninfected). Points are raw data grouped by helper presence.

3.4.2. Telomere dynamics and survival

Individuals with more negative ΔRTL values – indicating greater telomere shortening – had a greater subsequent risk of mortality (relative to individuals with no change in telomere length) while individuals with more positive ΔRTL values – indicating greater telomere lengthening – had a reduced risk of mortality (Fig. 3.4, Table 3.2). The quadratic function of ΔRTL was non-significant; thus, the effect of ΔRTL on mortality risk was constant throughout the range of ΔRTL. The association between mortality risk and ΔRTL was not dependent on the sex or age of these adult individuals (interaction terms reentered into final model; Table 3.2). As expected, older individuals had an increased mortality risk. Visual inspection of Schoenfield residuals showed no violation of the assumption of non-proportional hazards, meaning the effects of predictor variables on mortality risk were constant throughout the remaining lifespan.

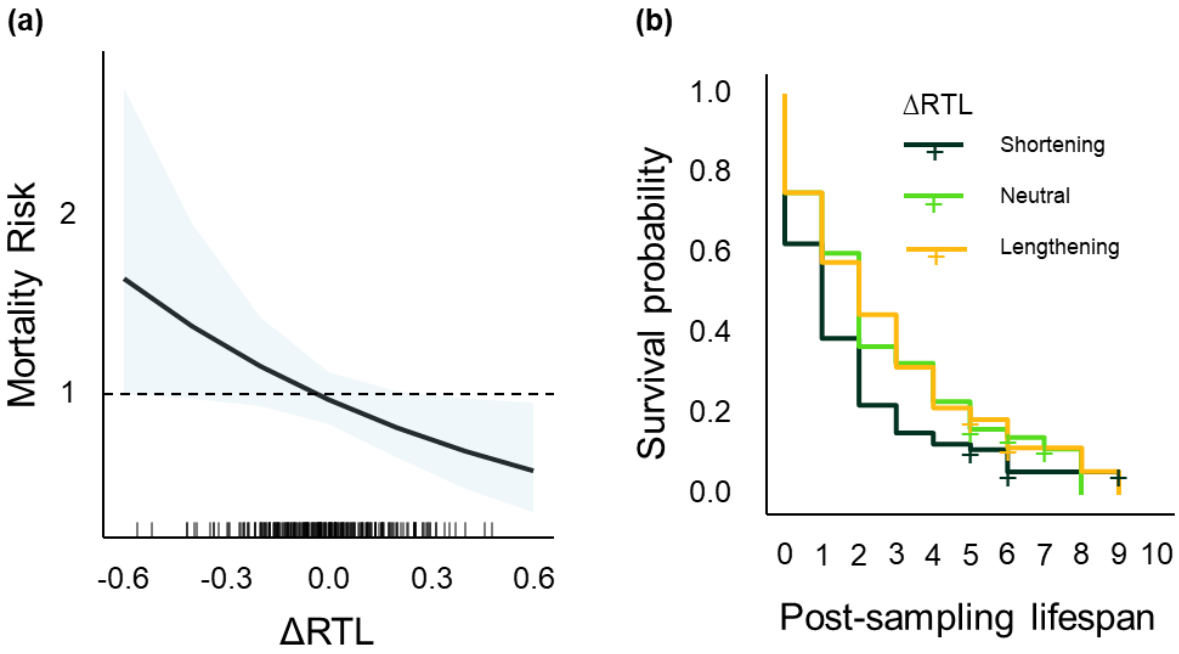

Figure 3.4: **(a)** The effect of ΔRTL change on mortality risk hazard ratios for Seychelles warblers. The reference hazard ratio (1 – dotted line) is set to a ΔRTL value of zero, with negative values indicating RTL shortening and positive values indicating RTL lengthening. The fit line represents the change in relative hazards – in this case risk of death – with the shaded area being 95% confidence limits. A mortality risk of 2 indicates that the risk of death is twice as high for the corresponding ΔRTL compared to the ΔRTL at 1. The bottom rug represents the distribution of ΔRTL values. **(b)** Survival curves for adult Seychelles warblers in relation to ΔRTL, which is split into tertiles that can be broadly characterised as RTL shortening, neutral (i.e. no change) and lengthening. Tick marks indicate individuals that were still alive at the end of our study period (n = 23). Units of post-sampling life-span is years.

Table 3.2: Cox proportional hazard model explaining variation in survival after the second of two samples was taken in relation to the change in telomere length (ΔRTL) between the two samples (*n* = 214). The reference category for sex is male.

Predictor	β	HR	*z*	*P*
ΔRTL	**-0.887**	**0.412**	**-2.153**	**0.031**
ΔRTL^2	-0.505	0.604	-0.356	0.722
Sex (Male)	0.047	1.048	0.323	0.747
Age	**0.051**	**1.052**	**2.206**	**0.027**
ΔRTL × Sex	-0.061	0.941	-0.072	0.943
ΔRTL × Age	-0.107	0.898	-0.939	0.348

3.5. Discussion

In our Seychelles warbler population, a change in telomere length (ΔRTL) was associated with life-history factors predicted to influence self-maintenance trade-offs - but only in females. Greater telomere shortening was observed in females with higher reproductive effort when living in areas of poorer food availability, as well as in individuals infected with malaria. Importantly, telomere lengthening was more often observed in females experiencing the reverse circumstance – i.e. those not infected with malaria and living in areas of high food availability – and those with helpers at the nest (the presence of which reduces reproductive effort per individual). These opposing changes in telomere length were not due to differences in initial telomere length or regression-to-the-mean effects. Consistent with ΔRTL being negatively correlated with stress, higher subsequent survival probabilities were associated with telomere lengthening, independently of sex and age.

3.5.1. Telomere dynamics

We found that telomeres shortened in individuals that tested positive for malaria, but only in females. This finding is consistent with previous studies demonstrating greater erythrocyte telomere shortening in malaria-infected individuals compared to uninfected individuals (Asghar *et al.*, 2015; Karell *et al.*, 2017). While we expected malaria to affect both sexes equally, sex-specific differences in the impact of malaria on telomere length have also been observed in blue tits (*Cyanistes caeruleus*; Sudyka, *et al.*, 2019). An emerging view is that telomere shortening is an outcome of immunological responses to infection (Giraudeau *et al.*, 2019). One such response – oxidative stress – is elevated in Seychelles warblers infected with malaria; albeit depending on the breeding stage (van de Crommenacker *et al.*, 2012). In our system, adults that test positive for malaria are most likely in the chronic (i.e. late) or relapse stages of the initial infection (first acquired as juveniles), or have been re-infected after clearing the initial infection (Valkiūnas, 2005). Therefore, the telomere shortening we observed seems to reflect a cost of persistent/re-emerging infection, perhaps due to immunological responses –rather than a direct cost of parasitism, which

tends to occur during the acute malarial stage (Asghar *et al.*, 2018). However, not knowing when malarial parasites became present or absent in blood, relative to the time of sampling, is a limitation of our observational study. Furthermore, while the frequency of recurring infection is generally low (ca. 20% within two years; Hammers *et al.*, 2016), initially uninfected individuals could have undetected outbreaks within the time period of repeated samples.

We also found that telomere shortening was greater in females that produced more offspring. Moreover, the relationship only occurred when the mean food availability was low during the period of offspring production. Food limitation is expected to increase reproductive effort per unit of reproductive success, and thus increase the costs of reproduction (Harshman and Zera, 2007; Santos and Nakagawa, 2012). For example, individuals in poor-quality territories may have to work harder to meet the food demands of offspring, leading to elevated stress (see Soulsbury and Halsey, 2018). Likewise, Seychelles warblers tend to be in poorer condition (in terms of oxidative stress and body mass) when provisioning chicks, compared to other nest stages, and when occupying poorer quality territories (Van de Crommenacker *et al.*, 2011, 2011). There are now several experimental and observational studies which show that individuals experiencing higher reproductive effort have shorter telomeres and/or experience greater telomere shortening (recently reviewed by Sudyka, 2019). However, few of these studies have explored associations between telomeres and reproduction in the context of food availability. Thus, our finding adds novel insight into life-history framework of telomere dynamics.

The relationship between telomeres and reproductive effort was only apparent in females. This was expected, since parental effort is higher in females; in the Seychelles warbler only females incubate and they also have higher provisioning rates than males (Hammers *et al.*, 2019; van Boheemen *et al.*, 2019). Thus, females benefit more from having nest helpers (Hammers *et al.*, 2019) and may be more responsive to differences in food availability when caring for offspring (e.g. Low *et al.*, 2012). Alternatively, telomere shortening may correlate with egg production – which is associated with substantial self-maintenance costs (Visser and Lessells, 2001; Williams, 2005) – more than with provisioning effort. Likewise, male telomere shortening may be more correlated with male-specific reproductive behaviours that were not accounted for in this study. For example, Bebbington *et al.* (2017) showed that Seychelles warbler males, which are more involved in territory defence, have more telomere shortening with increased competition from rival males.

Telomere length increased in females that produced more offspring when experiencing higher food availability, and when the production of offspring was assisted by nest helpers. This finding supports our main prior prediction – that telomere lengthening occurs in individuals experiencing lower levels of life-history stress. The non-biological explanation – that observed telomere lengthening is a consequence of high measurement error relative to attrition rate (Steenstrup *et al.*, 2013) – seems

unlikely for several reasons. First, the degree of within-individual telomere lengthening observed in our system is greater than that expected from measurement error alone (Spurgin *et al.*, 2018). Secondly, high measurement error and a lack of telomere shortening would result in no overall change (i.e. a random scatter of values around zero), whereas we observed an overall increase in telomere length consistent with our predictions. Lastly, our analysis accounted for regression-to-the-mean effects. This suggests that 'real' telomere lengthening (i.e. that which is not purely a consequence of measurement error) is more frequent in individuals with less stressful life-histories.

Telomere lengthening may be an outcome of lower reproductive costs associated with high food availability, cooperative breeding and absence of parasite infection. As discussed above, reduced reproductive effort is associated with the maintenance of longer telomeres and/or reduced telomere attrition. Some experimental studies that manipulated offspring number find no change or even slight telomere lengthening in treatment groups with the fewest offspring (Kotrschal, Ilmonen and Penn, 2007; Heidinger *et al.*, 2012; Sudyka *et al.*, 2014); however, these observations tend to be reported as reduced telomere shortening. Furthermore, reduced oxidative damage and telomere lengthening have also been observed in wild rodents receiving food supplements (Fletcher *et al.*, 2013; Hoelzl *et al.*, 2016). Thus, telomere lengthening may occur because plentiful food permits the allocation of energy to mechanisms involved in restoring previously lost telomere length, such as telomerase. This is a possibility in the Seychelles warbler, as high telomerase activity has been observed in the bone marrow (relative to other tissues) in adults of other bird species (Haussmann *et al.*, 2007).

In addition to reflecting the alleviated costs of reproduction in females with helpers and high food availability, telomere lengthening potentially may also be associated with intrinsic quality and/or condition (Bauch, Becker and Verhulst, 2013; Sudyka, 2019). Numerous studies have shown that telomere length is positively associated with individual quality and reproductive success (e.g. Pauliny *et al.*, 2006; Kasner *et al.*, 2013; Parolini *et al.*, 2017; Angelier *et al.*, 2019). Females which have higher reproductive success, territory quality and nest-helpers are likely to represent the best quality females in our population, and thus they may be better able to invest in mechanisms that restore/elongate telomere length, despite the potential for reproductive effort to shorten telomeres (Bauch, Becker and Verhulst, 2013). In contrast, females that produced few offspring as a consequence of poor intrinsic quality (rather than an effect of food availability or helper presence) may be unable to invest in such telomere-lengthening mechanisms, resulting in the observed lack of change or shortening of telomere length in these females. However, due to the limitations of observational studies, and the correlative nature of reproductive effort, reproductive success and associated factors (i.e. food availability, cooperative breeding), we are unable to separate the effects of life-history costs and intrinsic quality on telomere dynamics. Nevertheless, our study indicates that the telomere lengthening observed in some wild populations is not necessarily

random, or merely an artefact of measurement error, but can instead be associated with important life-history trade-offs and/or traits.

3.5.2. Telomere dynamics and survival

We found that individuals with greater telomere shortening had lower survival prospects. This finding is consistent with short telomeres being negatively associated with survival in the Seychelles warbler (Barrett *et al.*, 2013) and in a range of other wild vertebrate species (reviewed in Wilbourn *et al.*, 2018). Telomere shortening can directly impact survival by increasing the frequency of critically short telomeres, which can trigger cellular senescence (Kurz *et al.*, 2004). However, our measure of telomere length is a mean value (i.e. across chromosomes and cells) rather than a measure of the frequency of short telomeres *per se* (Bendix *et al.*, 2010). The non-causal explanation is that factors which shorten telomeres – such as oxidative stress – also cause wider cellular damage that ultimately increases mortality risk. Interestingly, we show that infection with malaria, for which we have not been able to find a survival impact in this species (Hammers *et al.*, 2016), may increase mortality risk via mechanisms that also shorten telomeres. Importantly, the telomere–survival relationship was not solely driven by negative effects (i.e. cellular damage and/or critically short telomeres), since individuals with lengthened telomeres had better survival prospects relative to individuals with no change in telomere length. This finding is consistent with the positive health and longevity effects of telomerase in mice (Bernardes de Jesus *et al.*, 2012; Simons, 2015) but contrasts with the results of Wood and Young, (2019), who found that increased telomere length was not associated with higher nestling survival in white-browed sparrow-weavers (*Plocepasser mahali*). In our study, telomere lengthening was also associated with life-history traits known to benefit survival and longevity in the Seychelles warbler: high food availability (Brouwer *et al.*, 2006) and helpers (Hammers *et al.*, 2019). This suggests that telomere lengthening may be characteristic of a strategy in which individuals make higher reproductive investments in more favourable environments, without incurring survival costs (as suggested by Hoelzl *et al.*, 2016).

3.5.3. Conclusion

Our study adds to the growing body of literature on the bidirectionality of within-individual telomere dynamics in ecological settings. We found that telomere lengthening can reflect good current environmental conditions and subsequently is linked to better survival prospects. Therefore, our study echoes conclusions from previous studies; that the within-individual variability and lengthening of telomeres means single measures of telomere length may not be a reliable indicator of damage accumulated in an individual's past life, nor their future performance – and hence not a good biological age marker. Future studies should determine the mechanisms behind telomere lengthening observed in wild populations, and whether telomere lengthening is coordinated across multiple tissue types within individuals.

3.6. References

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3.7. Supplements

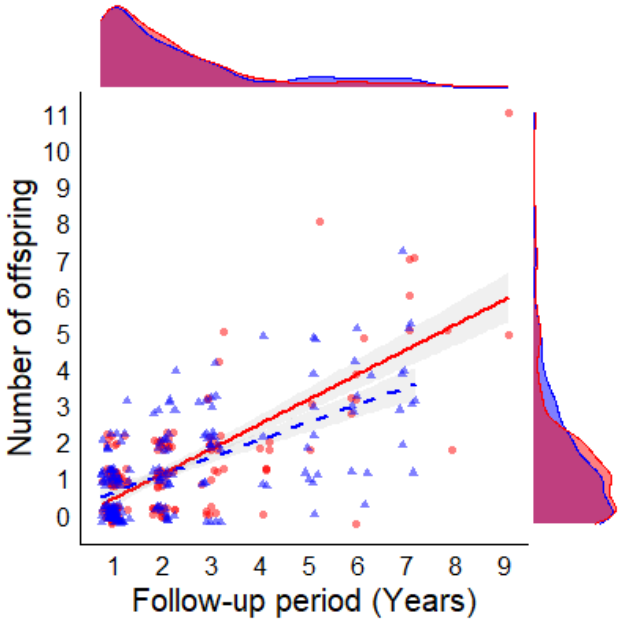

Figure S3.1. The relationship between offspring production by focal individuals (red = female; blue = male) and the duration of the period between pairs of repeated blood samples used for RTL analysis in the Seychelles warbler. Fit lines are linear regressions (±95% CI) between offspring number and follow-up years for males and females. Density plots show the frequency distribution of data.

Table S3.1. Linear mixed effects model explaining variation in ΔRTL (adjusted for the regression-to-mean-effect) between consecutive blood samples from the major breeding seasons in Seychelles warblers (males and females combined). The significance of helper presence and malaria effects depended on the inclusion of two-way interactions with sex.

Predictor	**β**	**SE**	***df***	***t***	***P***
(Intercept)	0.036	0.028	146.339	1.272	0.205
Sex (Male)	0.003	0.026	228.808	0.133	0.894
Food availability	0.016	0.015	244.333	1.043	0.298
Number of offspring	**-0.033**	**0.016**	**326.323**	**-2.106**	**0.036**
Helper presence (Yes)	**0.080**	**0.040**	**309.626**	**1.975**	**0.049**
Malaria (Yes)	**-0.062**	**0.029**	**334.446**	**-2.146**	**0.033**
ΔRTL period	-0.008	0.013	257.139	-0.656	0.513
Age	-0.007	0.004	322.438	-1.801	0.073
Sex × Food Availability	-0.024	0.019	319.548	-1.248	0.213
Sex × Number of offspring	0.031	0.020	333.174	1.522	0.129
Sex × Helper	-0.060	0.054	323.437	-1.105	0.270
Sex × Malaria	0.048	0.038	327.387	1.247	0.213
Food availability × Number of offspring	**0.041**	**0.015**	**340.126**	**2.810**	**0.005**
Sex × Food Availability × Number of offspring	**-0.049**	**0.020**	**329.982**	**-2.493**	**0.013**
Random Factors	**359 observations**		**Variance**		
Individual Identity	227 Individuals		0.002		
Catch year	18 years		0.001		
qPCR plate identity 1	69 plates		0.001		
qPCR plate identity 2	62 plates		0.004		
Residual			0.024		

Table S3.2: Linear mixed effects models explaining variation in ΔRTL (unadjusted for the regression-to-mean-effect) between consecutive blood samples from the major breeding seasons in female and male Seychelles warblers.

FEMALES				
Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.922	0.068	13.547	<0.001
Initial RTL	**-0.991**	**0.065**	**-15.353**	**<0.001**
Food Availability	0.017	0.015	1.120	0.266
Number of Offspring	**-0.033**	**0.016**	**-2.083**	**0.039**
Helper presence (Yes)	**0.081**	**0.037**	**2.176**	**0.031**
Malaria (Yes)	**-0.061**	**0.028**	**-2.199**	**0.029**
ΔRTL period	-0.002	0.017	-0.119	0.905
Age	-0.007	0.006	-1.337	0.183
Food × Offspring	**0.039**	**0.014**	**2.850**	**0.005**
Random factors	**170 observations**	**Variance**		
Individual identity	108 individuals	<0.001		
Catch year	18 years	0.001		
Plate 1 identity	54 plates	0.001		
Plate 2 identity	49 plates	0.003		
MALES				
Predictor	**β**	**SE**	***t***	***P***
(Intercept)	0.871	0.074	11.761	<0.001
Initial RTL	**-0.972**	**0.076**	**-12.821**	**<0.001**
Food Availability	-0.002	0.015	-0.134	0.894
Number of Offspring	0.001	0.018	0.079	0.937
Helper presence (Yes)	0.016	0.040	0.405	0.686
Malaria (Yes)	-0.007	0.030	-0.251	0.802
ΔRTL period	-0.019	0.018	-1.040	0.301
Age	-0.015	0.015	-1.022	0.308
Random factors	**189 observations**	**Variance**		
Individual identity	119 individuals	0.007		
Catch year	16 years	0.001		
Plate 1 identity	65 plates	0.001		
Plate 2 identity	56 plates	0.005		

Chapter 4
Seychelles warblers with silver spoons: juvenile condition is a lifelong predictor of annual survival, but not annual reproduction or senescence

As part of my PhD training I did an internship at the British Beet Research Organisation (BBRO) which implements and commissions research to support the UK sugar beet industry.

4.1. Abstract

The environment experienced during development, and its impact on intrinsic condition, can have lasting outcomes for adult phenotypes and could contribute to the individual variation in senescence trajectories. However, the nature of this relationship in wild populations remains uncertain, owing to the difficulties in summarizing environmental complexity and long-term monitoring of individuals from free-roaming long-lived species. In this study, we determine whether juvenile condition (derived from measures of body mass and size) is associated with senescence-related traits of a closely monitored population of Seychelles warblers (*Acrocephalus sechellensis*). Juveniles with a higher condition index were more likely to survive to adulthood – suggesting these juveniles experienced better developmental conditions. Furthermore, these juveniles as adults were in better condition and had higher rates of annual survival, independently of age. In contrast, there was no association between juvenile condition and declines in adult telomere length (a measure of somatic stress) or annual reproduction. These results indicate that juvenile condition, while not associated with senescence trajectories, can influence the likelihood of surviving to old age due to silver-spoon effects. This study shows that measures of intrinsic condition in juveniles can provide important insights into long-term fitness of individuals in wild populations.

4.2. Introduction

Senescence – defined as the decline in fitness-related traits with advancing age - is widespread across the tree of life (Jones et al, 2014). However, longitudinal studies have demonstrated that, even within the same species, individuals can show considerable variation in their onset and rate of senescence in natural environments (Williams *et al.*, 2006; Nussey *et al.*, 2013). Identifying drivers of this individual variation is vital for understanding the causes and evolution of senescence. Environmental factors can play a crucial role in shaping individual senescence (Monaghan *et al.*, 2008). For example, elderly individuals have been shown to be more vulnerable to harsh environments than prime-aged individuals (Reichert *et al.*, 2010; Oro *et al.*, 2014). Furthermore, the onset and rate of senescence can be affected by the environment experienced during early-life. The developmental period – the time from conception to sexual maturity – is of particular importance in modulating the adult phenotype (Lindström, 1999; Taborsky, 2006; Vaiserman, Koliada and Lushchak, 2018). However, there remains uncertainty on how the developmental environment affects senescence.

There is abundant evidence that beneficial (or benign) environmental conditions during development, such as a high nutrition diet, have positive effects on multiple aspects of individual fitness (Lindström, 1999; Cooper and Kruuk, 2018a); a phenomenon referred to as the “silver-spoon effect” (Monaghan, 2008a). The silver-spoon effect may result in delayed (or reduced rate of) senescence when the fitness benefits of beneficial conditions during development persist into late-

life (Nussey *et al.*, 2007; Pigeon, Festa-Bianchet and Pelletier, 2017; Cooper and Kruuk, 2018). However, silver-spoon effects may also lead to earlier and accelerated senescence (e.g. Hunt *et al.*, 2004; Spagopoulou *et al.*, 2020), for example when a greater allocation of resources into growth and reproduction during early-life - as a result of beneficial early-life conditions - negatively affects somatic maintenance and, consequently, later-life fitness (Hammers *et al.*, 2013; Lemaitre *et al.*, 2014, Kirkwood, 1977). A recent meta-analysis found that good developmental environments are more often associated with slower rates of reproductive (but not survival) senescence in wild populations; suggesting persistent silver-spoon effects are more prevalent (or detectable) than early- versus late-life fitness trade-offs (Cooper and Kruuk, 2018).

While development environments clearly act as a constraining force on an individual's age-specific fitness (and hence senescence), this may also interact with the responses of juveniles to those environments. For example, harsh developmental environments can generate more resilient adult phenotypes ('thrifty phenotype hypothesis'; Hales and Barker 2001) or remove individuals with less-resilient phenotypes at younger ages (selection hypothesis; Nol and Smith, 1987), resulting in individuals that are more resistant to fitness declines in late-life (Marshall *et al.*, 2017). Additionally, the developmental environment, whether good or bad, may be less important for senescence than whether or not the same environment persists into adulthood, with environmental mismatches resulting in poorly-adapted, faster senescing individuals (Gluckman, Hanson and Spencer, 2005; Monaghan, 2008). Thus, while there is agreement that developmental environments can explain between-individual variation in senescence, there is yet little consensus on the reason for, or direction of, the effect.

Determining the relationship between a developmental environment and senescence is not straightforward, not least because of the multi-faceted nature of environments. Most previous studies have measured characteristics of the environment directly, with proxies of food availability or closely related factors e.g. population density and weather (Cooper and Kruuk, 2018a). However, such measures often lack the resolution to determine local individual-level environments, nor do they reflect the developmental decisions occurring within juveniles in response to environmental stimuli. This difficulty can be addressed by measuring metrics of intrinsic condition that reflect the developmental environment.

Body mass and derived indices (e.g. size-adjusted mass) are commonly used measures of individual condition in ecological studies. Body mass is strongly positively correlated with body fat content – the main component of energy storage – and the structural size of individuals (Schulte-Hostedde *et al.*, 2005; Hayes and Shonkwiler, 2010; Labocha and Hayes, 2012a). In juveniles, being heavier or larger often reduces vulnerability to predation (at least in non-flying organisms – see below), food-shortages and cold-weather events, and can provide a competitive advantage over peers (Arendt,

1997). As a result, juvenile body mass is generally positively associated with survival to adulthood in birds and mammals (Tinbergen and Boerlijst, 1990; Ronget *et al.*, 2018a). However, there has been extensive debate concerning the quantification of condition (Green, 2001; Speakman, 2001; Stevenson and Woods, 2006; Labocha and Hayes, 2012b; Labocha, Schutz and Hayes, 2014; Wilder, Raubenheimer and Simpson, 2016; Frauendorf *et al.*, 2021), which agree that simple positive correlations between body mass, condition and fitness cannot be assumed without validation. For example, excessive fat deposition (i.e. high body mass relative to size) can reduce an individual's ability to evade predators, especially for flying organisms (Gosler, Greenwood and Perrins, 1995; Covas *et al.*, 2002). Therefore, the optimal body mass is expected to be less than the maximum achievable body mass (Barnett *et al.*, 2015).

Juvenile body mass and derived condition indices may also have lasting associations with fitness-related traits (e.g. Merilä and Svensson, 1997). The growth and fat deposition of juveniles can be constrained or delayed by poor (e.g. nutrient-limited) environments, with long-lasting consequences for physiological development and fitness (Metcalfe and Monaghan, 2001; Hsu, Dijkstra and Groothuis, 2017; Seress *et al.*, 2020). For example, in captive zebra finches (*Taeniopygia guttata*) juveniles reared on poor-quality diets had lower body mass, but also lower reproductive success (Haywood and Perrins, 1992; Blount *et al.*, 2006) and shorter adult life-spans (Birkhead, Fletcher and Pellatt, 1999). Therefore, juvenile body mass can reflect a silver-spoon effect of early-life environment on adult fitness. Conversely, due to trade-offs between early and late-life fitness, individuals which are larger (and thus heavier) or grow at faster rates can have reduced fitness in late-life (Miller *et al.*, 2002; Metcalfe and Monaghan, 2003; Kraus, Pavard and Promislow, 2013; Vaiserman, Koliada and Lushchak, 2018). Achieving larger absolute size, or attaining adult size earlier, reflects a greater investment in growth, which may incur costs in terms of late-life fitness i.e. a 'live-fast-die-young' phenotype. Likewise, individuals reared in poor-nutrition environments may prioritize energy retention (i.e. fat content) over growth and size i.e. a 'thrifty' phenotype. Therefore, the initial benefits of high body mass, large size or a beneficial early-life environment (e.g. high food abundance) can result in accelerated senescence.

Our current understanding of the relationships between juvenile body mass, size and senescence is mainly restricted to studies that have manipulated body mass or growth rates of laboratory and captive populations (but see Spagopoulou et al., 2020). In wild populations, body mass and derived condition indices are more often related to immediate fitness (i.e. annual measures of survival and reproductive success) rather than lifetime or late-life fitness; owing to the difficulty of monitoring individuals across their entire life course in many wild populations (but see Lewin *et al.*, 2017). Therefore, it is not certain whether natural variation in juvenile body mass and size can explain variation in senescence trajectories observed in wild populations. In this study, we determine whether the body mass of juveniles predicts fitness-related traits in adult Seychelles warblers,

Acrocephalus sechellensis – a small insectivorous passerine endemic to the Seychelles. The closely monitored population on Cousin Island is uniquely suited for this study; each individual has annual measures of survival and reproduction, and repeated measures of condition starting from juvenile age. This study will contribute to our understanding of the role that early-life condition plays on variable senescing phenotypes in wild populations.

4.3. Methods

4.3.1. Study species and data collection

The Seychelles warbler is a small insectivorous passerine endemic to the Seychelles. The population on Cousin Island (29 ha; 4°20' S, 55°40' E) – containing *ca.* 320 adult individuals at any given point (Brouwer et al 2009) – has been extensively monitored since 1985 (Komdeur, 1992; Hammers *et al.*, 2015; Sparks *et al.*, 2020a). Since 1997, nearly all individuals (>96%) have been ringed with a unique combination of a British Trust for Ornithology (BTO) metal ring and three colour rings for easy identification (Richardson *et al.*, 2001; Raj Pant *et al.*, 2020a). Individuals are usually first caught as nestlings, or as dependent juveniles (<5 months old) in their natal territory using mist nets (see Kingma *et al.*, 2016 for details). Juveniles are aged as fledglings (1–3 months), old fledglings (3–5 months) or sub-adults (5–12 months) based on behaviour and eye colour (Komdeur, 1992). In addition to capturing unringed juveniles, as much of the ringed adult population as possible (normally ca. 35%) is re-captured and sampled during the major breeding season (June–September) each year.

The population is structured into ca. 115 clearly defined territories (Kingma *et al.*, 2016), each containing a socially monogamous dominant pair. However, the Seychelles warbler is a facultative cooperative breeder; thus, ca. 50% of territories contain 1-5 sexually mature subordinates (usually, but not always, past offspring of the dominant pair), of which ca. 20% of males and ca. 42% of females engage in helping behaviour and cobreeding (Richardson, Burke and Komdeur, 2002; Hammers *et al.*, 2019). Each year, during the major breeding season, each territory is visited at least every two weeks to identify all individuals present and determine their status through behavioural observations (Richardson, Burke and Komdeur, 2003). During visits, the dominant female is followed for ≥ 15 minutes to assess breeding activity (Richardson, Burke and Komdeur, 2007). The majority of breeding activity (94% of territories) occurs from June to August, but a minor breeding season also occurs from January to March (Komdeur and Daan, 2005). Most breeding attempts involve one-egg clutches (Komdeur, 1994a) but clutches of two or three eggs occur (Richardson *et al.*, 2001). The extensive duration of parental care (ca. three months post-fledging), relative to the length of breeding seasons, limits the opportunity for multiple successful breeding attempts

(Komdeur, 1996b). As a result, the vast majority of successful territories produce just one offspring per breeding season.

In both males and females, annual reproductive success follows a bell-shaped relationship with age; increasing until 7–8 years-of-age before declining in older age (Hammers *et al.*, 2012; Raj Pant *et al.*, 2020a). The resighting probability of adults during the major breeding season is close to one (0.98 ± 0.01 SE; Brouwer *et al.*, 2010) and dispersal from the island is virtually absent (Komdeur *et al.*, 2004). Therefore, individuals that are not observed during the major breeding season can be confidently assumed dead. First year survival is 0.61 ± 0.09 SE, increasing to a relatively stable 0.84 ± 0.04 SE annual survival in adults (Brouwer *et al.*, 2006), before declining from ca. 7 years of age i.e. the onset of survival senescence (Hammers *et al.*, 2013; 2015). In elderly females, reproductive success is also lower in the last year of life ("Terminal year effect"), suggesting that elderly females are in poorer physiological condition prior to death (Hammers *et al.*, 2012).

During capture events, body mass is measured using either a Pesola or electronic scale (± 0.1g) and structural size is measured using sliding callipers (± 0.1mm) as the length of the right tarsus. *Ca* 25 ul of blood is taken from the brachial vein and stored in 100% ethanol (Richardson *et al.*, 2001). DNA extracted from blood samples (following Richardson *et al.*, 2001) is used to confirm sex, using up to three sexing markers, and assign parentage using MasterBayes 2.52 (Hadfield et al 2006) based on genotypes derived from 30 microsatellite loci (for details see Sparks et al., 2020). Relative Telomere Length (RTL; the concentration of amplified telomeric DNA relative to that amplified at GAPDH – a single copy gene) has also been measured as part of a previous study (for details see Spurgin *et al.*, 2017). In many species, including the Seychelles warbler, telomere length declines with age and with increased exposure to various stressors (Barrett *et al.*, 2013; Spurgin *et al.*, 2017; Young, 2018). Thus, telomere length has been advocated as a marker of accumulated somatic stress and survival prospects (Wilbourn *et al.*, 2018).

4.3.2. Statistical analysis

All analyses were performed in Rstudio (version 1.2.5033 and R version 4.0.3, Rstudio Team, 2020). We selected all individuals with biometric data at post-fledging juvenile age (3 weeks to 5 months after hatching). This is just after the developmental period when skeletal growth is complete (Komdeur, 1991), when juveniles are still dependent on the adults from the natal territory, and before sexual maturity (ca. 8 months; Komdeur, 1997). The Seychelles warbler is sexually dimorphic, with males being larger than females (Richardson 2013). Body mass, as well as being higher in males than females, is also positively correlated with structural size (tarsus length) and the time of day of capture (Fig. S4.1, Table S4.1,Kingma *et al.*, 2016). Using linear mixed effect models, which predicted the mass of an individual for a given tarsus length, measured at a given time of day, we calculated residual mass (i.e. the difference between observed and predicted mass)

separately for males and females. Observer was included as a random effect to control for possible observer bias in measurements. This approach eliminates the dependency of body mass on other predictors, namely tarsus length and sex, in multiple regression models; thus, giving the "true" effect of body mass on the response variable beyond that caused by size- or sex-related differences in body mass (e.g. Ross *et al.*, 2021). Residual mass (hereafter, "condition") is a widely used condition index that is highly correlated with fat content in other species (Schulte-Hostedde *et al.*, 2005; Labocha and Hayes, 2012a). However, condition can also reflect differences in bodily components other than fat content, such as muscle and organ mass (Labocha and Hayes, 2012b; Frauendorf *et al.*, 2021). For a subset of juveniles (N = 364), we had visual estimates of abdominal fat (hereafter, "fat scores") which, although positively correlated (β = 0.105 ± 0.050, t = 2.088, P = 0.038; Fig. S4.2), explained a negligible amount of variation in condition (adjusted R^2 = 0.009). Since we lack the data to test the degree to which condition and/or fat scores correlate with actual fat content in this species, condition is more broadly defined as the mass of an individual that is independent of structural size, sex and capture time. All analyses were repeated with raw body mass instead of condition, which produced qualitatively similar results (not shown).

We first determined whether juvenile condition influenced survival to adulthood (>1 year of age). Survival to adulthood (yes/no) was fitted as a binomial response with log link function in a generalized linear mixed model (GLMM) using lme4 1.1-25 (Bates *et al.*, 2015). Condition was entered as main effect and as a quadratic (i.e. squared) function (see Barnett *et al.*, 2015). To confirm the fit suggested by the quadratic function, we repeated the analyses using a Generalized Additive Mixed Model (GAMM) using gamm4 (v0.2-6; Wood, 2017) with a non-parametric smoothing spline for juvenile condition. Additional predictors included sex, age (months) of measurement and tarsus length (mean-centered by sex) - to determine whether skeletal size influences survival independently of condition. Year was included as a random factor to account for annual differences in juvenile survival.

In subsequent juvenile survival models, we included information on the individual's overall and immunological genetic diversity (for which we had a reduced dataset). Heterozygosity, MHC diversity (log-transformed) and the presence of TLR3^{A} and MHC *Ase-ua4* alleles (yes/no) have been positively associated with juvenile survival in earlier studies on this species (Richardson, Komdeur and Burke, 2004; Brouwer *et al.*, 2010; Davies *et al.*, 2021). Therefore, by including these additional predictors into our model, we determined whether the juvenile survival-condition relationship occurred independently of these genetic effects.

Secondly, for juveniles that survived to adulthood, we tested whether juvenile condition was associated with two measures of adult condition – body mass and RTL – to assess physiological senescence. Both traits were fitted as responses in two Linear Mixed Models (LMM). Juvenile

condition, adult tarsus length and sex were included as main effects. In the body mass model, capture time was included as an additional predictor. We opted to use raw body mass as a measure of adult condition, rather than the residual condition index used in juveniles, since these measures are equivalent in a model controlling for the effects of sex, tarsus length and capture time on adult body mass in a LMM (Freckleton 2002). In the RTL model, we included technician as a two-level factor to account for technician-related differences in RTL (Sparks *et al.*, 2020b). We used within-subject centering (van de Pol and Wright, 2009) to separate the role of between- versus within-individual variation with age, i.e. cross-sectional from longitudinal effects. In this way, the individual's age (at measurement of body mass/telomere length) was split into two predictors, (i) mean age across all sampling events for a given individual (mean age), and (ii) within-individual deviation from mean age (Δ age). An interaction term between juvenile condition and Δage tested whether juvenile condition alters the within-individual slope of adult body mass/telomere length. Since individuals often had multiple measures of adult body mass and telomere length, individual identity was included as a random effect. In the body mass model, observer was also included as a random effect to control for possible observer bias in measurements. In the telomere length model, PCR plate identity was included as a random effect to control for possible inter-plate variation in telomere length (Sparks *et al.*, 2020b).

Thirdly, we tested whether juvenile condition was associated with two fitness components shown to senesce in later adult life in the Seychelles warbler; annual survival and annual reproduction (Hammers *et al.*, 2012, 2013, 2015; Raj Pant *et al.*, 2020a). For this analysis, we excluded individuals that had not died by the end of the study period (2019). Furthermore, we excluded the first year of the individual's life, since first year survival was covered in our survival to adulthood analysis (see above) and individuals rarely reproduce before one year of age (Komdeur, 1991, 1992). Annual survival was defined as whether or not the individual died before the subsequent main breeding season. Annual reproduction indicated whether the individual produced at least one independent offspring (i.e. surviving to at least 5 months of age) during that year. These fitness traits were fitted as binomial responses (yes versus no) with a log link function in GLMMs. Juvenile condition was entered as a main effect and as an interaction term with age. A significant main effect would indicate that juvenile condition influences the fitness component overall, independently of age, while a significant interaction would indicate that juvenile condition modifies the age-dependent change in the fitness component. Age (at the end of the main breeding season) was included as a linear and squared term, (Hammers *et al.*, 2012,Raj Pant *et al.*, 2020a). To confirm the presence of late-life declines in survival and reproduction, we repeated analyses including only data above the age of onset of declines; determined visually from non-standardized squared functions of age (Fig. 4.3a and 4.4a). Sex and tarsus length were included as additional predictors. Since individuals had

multiple measures of fitness, individual identity was included as a random factor. Year was also included as a random factor to control for annual differences in fitness (Brouwer et al., 2006).

In the annual reproduction model, additional predictors were included due to their previously reported associations with annual reproduction and fledging success in this system. An interaction term between sex and age was included due sex-specific differences in the onset of reproductive senescence (Hammers *et al.*, 2012; Raj Pant *et al.*, 2020b). Year quality (i.e. island-wide mean insect abundance during the years main breeding season) and territory quality (i.e. the difference between year quality and insect abundance within the individual's territory) data were available for some years (all except 2000 – 2002 and 2005) and were included due to positive associations with fledging success (Hammers *et al.*, 2012). Whether or not the year in question was the last year of an individual's life (terminal year, yes/no) was included, since fledging success was found to be lower in the terminal year of old (≥ 6 years) females (Hammers *et al.*, 2012). We also included age-at-death, to quantify the within-individual effect of age on reproductive success while controlling for selective disappearance (van de Pol and Verhulst, 2006, Hammers et al. 2012).

In all models, non-significant interaction terms were removed sequentially (in order of least significance), so that the first-order effects could be interpreted, and were only reported if of specific interest. All fixed effects remained in final models (regardless of significance) except for squared functions of continuous variables, which were removed when non-significant (see Whittingham *et al.*, 2006). Parameter estimates and significance of removed effects were determined by re-entering them into final models. Continuous fixed effects involved in squared effects and interactions were mean-centered to reduce collinearity and aid interpretation (Schielzeth, 2010). Where model singularity errors occurred, we applied maximum a posteriori estimation using blme (v1.0-5; Dorie, 2013). To aid model convergence of GLMMs, we used the "BOBYQA" nonlinear optimization (Powell, 2009). Model fit was calculated as conditional R^2 using MuMin (v1.43.17; Bartoń, 2019).

4.4. Results

4.4.1. Juvenile survival

Of the 711 juveniles included in our analysis, 545 survived to adulthood (>1 year-of-age). Juveniles with a higher condition index were more likely to survive to adulthood (Table 4.1; Fig. 4.1). The squared condition term revealed a 'ceiling effect'; juvenile survival increased with condition at values < 0, above which juveniles shared the highest survival prospects (ca. 80%; Fig. 4.1). The non-parametric smoothing function for condition from the GAMM showed a very similar pattern compared to the quadratic condition term shown in Fig. 4.1 (df = 2.618, F = 5.38, P = 0.001; Fig. S4.3). Juvenile survival also increased with tarsus length, but was not associated with age or sex

(Table 4.1). Complete data for genetic factors (heterozygosity, MHC diversity, TLR3A and MHC Ase-ua4 allele presence), previously shown to influence juvenile survival, were available for 240 juveniles. In our model, none of these genetic factors were associated with survival (Table S4.2) and the observed juvenile survival-condition relationship remained significant while controlling for these genetic factors (Table S4.2).

Table 4.1: General linear mixed effects exploring predictors of juvenile survival to adulthood in Seychelles warblers. Significant effects are in bold.

Juvenile survival; conditional R^2 = 0.061				
Predictor	**Estimate**	**SE**	***z***	***P***
(Intercept)	1.159	0.307	3.769	<0.001
Condition	**0.243**	**0.093**	**2.613**	**0.009**
Condition²	**-0.115**	**0.058**	**-1.989**	**0.047**
Age (months)	0.996	1.077	0.925	0.355
Sex (female)	-0.086	0.184	-0.469	0.639
Tarsus length	**0.208**	**0.095**	**2.195**	**0.028**
Random	**699 individuals**	**Variance**		
Catch year	22 years	0.048		

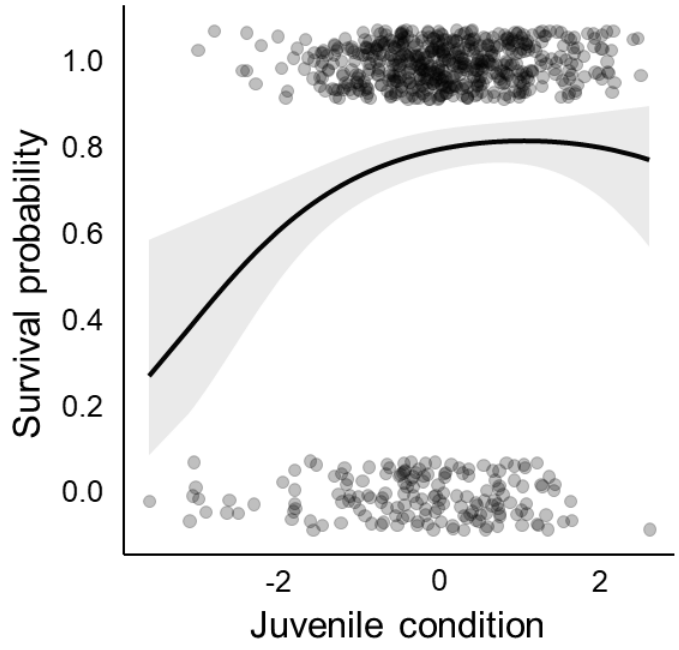

Figure 4.1: The probability of juvenile Seychelles warblers surviving to adulthood (>1 year of age) relative to their juvenile condition index. The solid line is a binomial regression between survival (Y/N) and condition with 95% confidence limits. Raw data points depict the distribution of jittered observed survival counts (1 = survived, 0 = deceased).

4.4.2. Adult Body mass

As expected, adult body mass was higher in males and, in both sexes, increased with tarsus length and time of capture; in a similar manner to that observed with juvenile body mass (Table S4.1). While controlling for these factors, adult body mass was positively correlated with juvenile condition (Table 4.2a; Fig. 4.2). This indicated that relatively heavier or lighter juveniles tended to remain relatively heavier or lighter, respectively, as adults. Adult body mass increased with age between individuals (i.e. cross-sectional) and not within-individuals (longitudinal), but these slopes did not significantly differ (t = 1.776, P = 0.076), indicating that the between-individual rate of increase was not greater than the within-individual lack of change. (Table 4.2a).

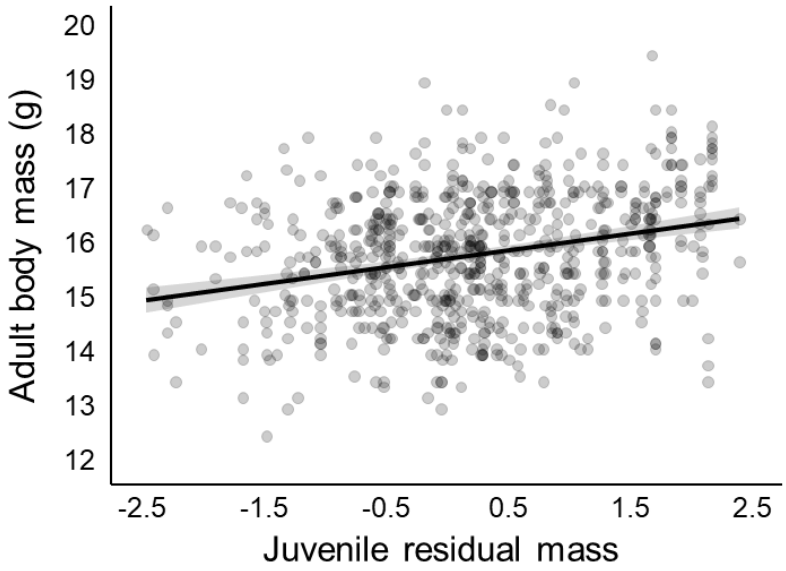

Figure 4.2: The relationship between juvenile condition and adult (>1 year of age) body mass in the Seychelles warbler. The fit-line is a linear regression with 95% confidence limits. Points depict raw data.

4.4.3. Telomere length

RTL tended to decrease both within and between individuals with increasing age (Table 4.2b). The within- and between individual rate of change in RTL did not significantly differ (t = -1.770, P = 0.078). Juvenile condition was not associated with overall adult RTL (Table 4.2b) nor the within-individual decline in RTL (Table 4.2b; Δ age × Juvenile condition). Telomere length was not associated with sex or tarsus length (Table 4.2b).

4.4.4. Annual adult survival

Annual survival remained relatively stable at ca. 80% from one to 7 years-of-age, beyond which annual survival declined with age (Fig. 4.3a). This decline in annual survival was confirmed by re-running the analysis with data ≥ 7 years-of-age (β = -0.2523 ± 0.086, z = -2.954, P = 0.003). Juvenile condition was positively associated with annual survival, independent of age (Table 4.3a; Age × Juvenile condition, Fig. 4.3b). Therefore, individuals with a higher condition index as juveniles had higher annual survival throughout adult life. Annual survival was not influenced by sex or tarsus length (Table 4.3a).

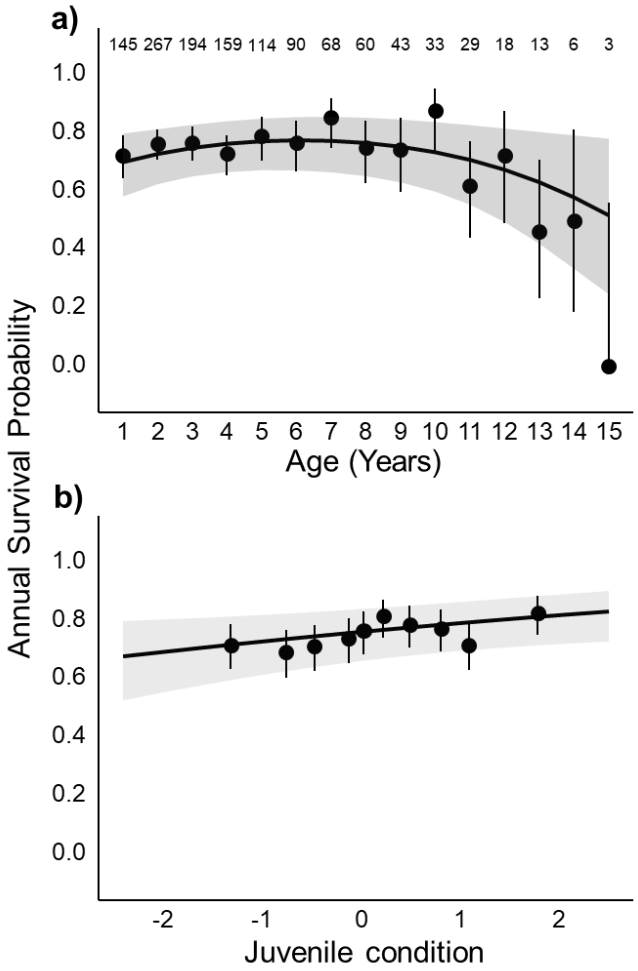

Figure 4.3: The probability of adult Seychelles warblers surviving to the next year relative to (a) age and (b) juvenile condition. The fit-lines are model-predicted survival curves with 95% confidence limits. Points with error bars are mean survival and binomial 95% confidence intervals of raw data, grouped by (a) age and (b) percentiles of juvenile condition; note that the x-axis position of points corresponds to the percentile distribution of juvenile condition. In text numbers in panel (a) refer to sample sizes per age.

4.4.5. Annual reproductive success

Neither year quality nor territory quality were associated with annual reproduction and so were removed as predictors to maximize sample size (N = 1034 versus N = 1242). Annual reproduction exhibited a humped relationship with age; increasing in early-life before peaking and declining from mid- to late-life (Fig. 4.4a). The age of the peak in annual reproduction (and thus the onset of reproductive senescence) differed between sexes (Fig. 4.4a), with female and male annual reproduction peaking at ca. 6 and 8 years-of-age. Annual reproduction was also lower in the terminal year (Table 4.3b). Re-running the analysis on ages from the onset of reproductive senescence (≥ 6 years for females, ≥ 8 years in males) confirmed that annual reproduction declined with advanced age, and that the slope of the decline was greatest in the terminal year (Age × Terminal year: $\beta = 0.442 \pm 0.180$, $z = 2.459$, $P = 0.014$, Fig. 4.4b). Juvenile condition was not associated with annual reproduction, nor with the age-dependent change in annual reproduction

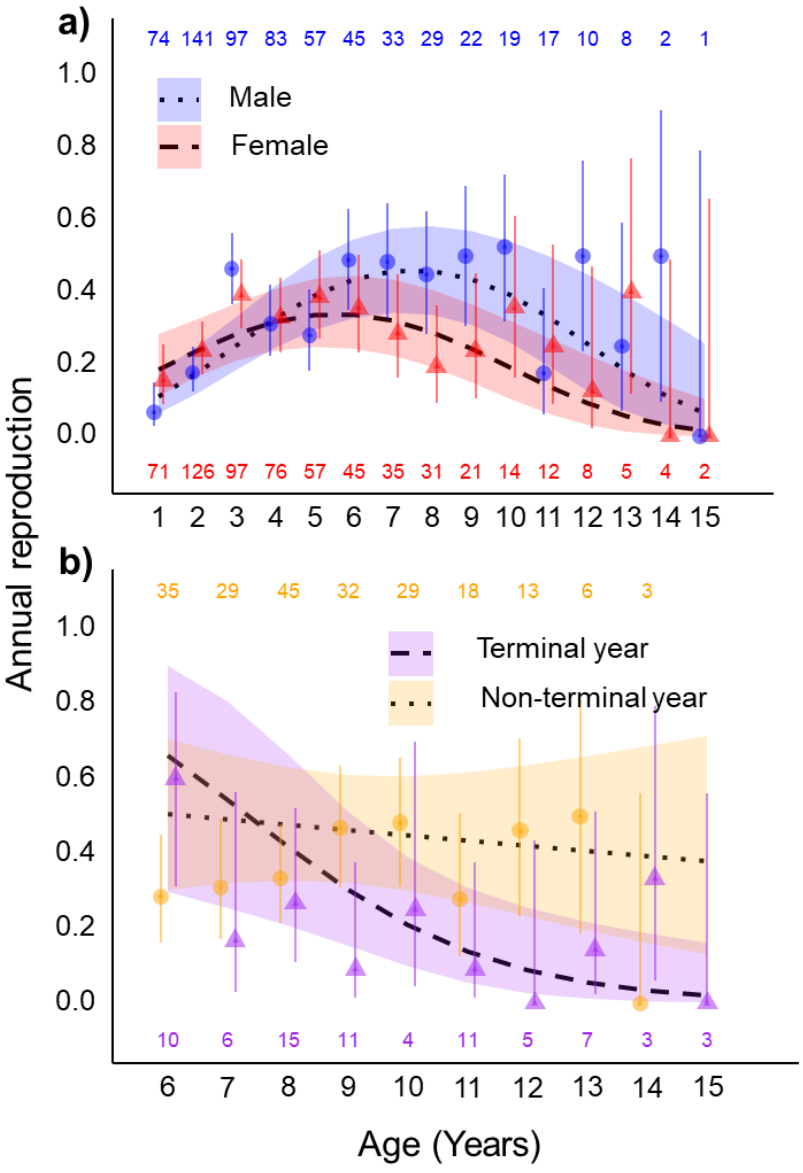

Figure 4.4: The probability of adult Seychelles warblers producing an independent offspring in a year relative to age and (a) sex and (b) terminal year (yes/no). The fit-lines are model-predicted probability curves with 95% confidence limits. Points with error bars are mean offspring and binomial 95% confidence intervals of raw data, grouped by age per sex (a) and age per terminal year (b). In text numbers refer to the sample sizes per age per grouping variable. Males and females had differing onsets of decline in annual reproduction (a), and the rate of decline was greatest in the terminal year (b).

(Table 4.3b; Age × Juvenile condition). Annual reproduction was not influenced by tarsus length (Table 4.3b).

Table 4.2: Linear mixed effects models explaining variation in a) Adult body mass, and b) Relative telomere length in the Seychelles warbler. Significant effects are in bold.

a) Adult body mass; conditional R^2 = 0.612				
Predictor	**Estimate**	**SE**	***t***	***P***
(Intercept)	7.107	1.288	5.520	<0.001
Juvenile condition	**0.199**	**0.036**	**5.573**	**<0.001**
Sex (female)	**-0.848**	**0.102**	**-8.315**	**<0.001**
Tarsus length	**0.338**	**0.050**	**6.800**	**<0.001**
Time of capture	**0.001**	**0.000**	**4.769**	**<0.001**
Mean age	**0.041**	**0.018**	**2.264**	**0.024**
Δ age	-0.003	0.016	-0.191	0.848
Δ age × Juvenile condition	-0.002	0.016	-0.105	0.917
Random	**704 observations**	**Variance**		
Bird Identity	311 individuals	0.114		
Observer	41 observers	0.040		
Residual		0.490		
b) Relative telomere length; conditional R^2 = 0.178				
Predictor	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.933	0.025	37.666	<0.001
Juvenile condition	0.009	0.010	0.933	0.353
Sex (female)	<0.001	0.019	0.000	1.000
Tarsus length	-0.002	0.010	-0.185	0.854
Δ age	-0.011	0.006	-1.770	0.078
Mean age	**-0.009**	**0.005**	**-1.987**	**0.049**
Technician	**0.081**	**0.022**	**3.709**	**<0.001**
Δ age × Juvenile condition	0.007	0.006	1.163	0.246
Random	**427 observations**	**Variance**		
Bird Identity	207 individuals	0.001		
qPCR plate	70 PCR plates	0.004		
Residual		0.032		

Table 4.3: General linear mixed effects models explaining variation in a) annual survival and b) annual reproductive success in adult Seychelles warblers. Significant effects are in bold.

a) Annual survival; conditional R^2 = 0.245				
Predictor	**Estimate**	**SE**	***z***	***P***
(Intercept)	1.176	0.252	4.674	0.000
Age	0.148	0.117	1.260	0.208
Age²	**-0.132**	**0.059**	**-2.239**	**0.025**
Juvenile condition	**0.175**	**0.080**	**2.185**	**0.029**
Sex (female)	0.066	0.147	0.449	0.653
Tarsus length	0.112	0.076	1.477	0.140
Age × juvenile condition	0.079	0.103	0.763	0.445
Age² × Juvenile condition	-0.077	0.065	-1.186	0.236
Random	1242 observations	Variance		
Bird Identity	306 individuals	0.110		
Year	21 years	0.897		
b) Annual Reproductive success; conditional R^2 = 0.287				
Predictor	**Estimate**	**SE**	***z***	***P***
(Intercept)	-1.136	0.261	-4.350	0.000
Age	**0.855**	**0.173**	**4.935**	**<0.001**
Age²	**-0.417**	**0.093**	**-4.501**	**<0.001**
Juvenile condition	0.114	0.095	1.197	0.231
Sex (female)	-0.143	0.210	-0.680	0.496
Tarsus length	-0.026	0.090	-0.289	0.773
Terminal year (no)	**0.718**	**0.210**	**3.415**	**0.001**
Age at death	0.052	0.131	0.398	0.691
Age × Sex (female)	**-0.597**	**0.205**	**-2.913**	**0.004**
Age² × Sex (female)	0.070	0.134	0.523	0.601
Age × Juvenile residual mass	0.094	0.113	0.828	0.408
Age² × Juvenile residual mass	-0.129	0.072	-1.794	0.073
Random	1242 observations	Variance		
Bird Identity	306 individuals	0.485		
Year	21 years	0.359		

4.5. Discussion

Juvenile condition (size-, sex- and time-corrected mass) was positively associated with survival to adulthood, independently of specific genetic factors. For individuals that survived to adulthood, juvenile condition was positively associated with adult body mass - indicating that individual differences in condition are maintained from the juvenile period to throughout adulthood. More importantly, the survival benefit of high juvenile condition persisted throughout adult-life. Therefore, juveniles that reached adulthood despite a low condition index still had poorer survival

in a given year compared to adults that had a high juvenile condition index. The effect of juvenile condition on annual survival was constant with age, i.e. the age-dependent decline in survival from 7 years-of-age observed in this species did not change in respect to juvenile condition. So while juveniles in better condition are more likely to reach older ages (i.e. have greater longevity), they still exhibit the same pattern of survival senescence as those individuals that had poor condition as juveniles. There was no effect of juvenile condition on annual reproductive success, nor the maintenance of adult telomere length.

That condition is positively associated with a juvenile's likelihood of surviving to adulthood in the Seychelles warbler is consistent with findings across birds and mammals (reviewed in Ronget *et al.*, 2018). A high condition index is likely to be indicative of a good natal environment, which subsequently leads to higher survival. Indeed, juvenile Seychelles warblers receiving cooperative nest care have higher provisioning rates and, consequently, higher mass than juveniles without helpers (Komdeur, 1994b; van Boheemen *et al.*, 2019). Furthermore, the presence of helpers and higher food abundance has been associated with higher juvenile survival (Komdeur, 1992; Brouwer *et al.*, 2006; Hammers *et al.*, 2021). While these findings are correlative, experimental studies demonstrate that manipulations of natal/early-life environments have similar outcomes for juvenile mass/condition and survival in other species (e.g. Le Galliard, Ferrière and Clobert, 2005; Grace *et al.*, 2017). Direct benefits of high condition also occur where this reflects more abundant energy stores (i.e. fat and protein), since newly independent juveniles lacking experience can be more vulnerable to starvation and exposure (e.g. Jones *et al.*, 2017). In other systems, the benefits of energy storage may be traded against increased predation risk, resulting in a condition-survival relationship that becomes negative with increasing condition (Adriaensen *et al.*, 1998; Blums *et al.*, 2005). In contrast to these systems, we found a condition-survival relationship did not become negative, which was expected given that post-fledging predation does not occur in this population (Komdeur, 1996a). In fact, previous work in this species suggests that individual condition (size-adjusted mass) is primarily constrained by food availability and population density (Brouwer *et al.*, 2009). Therefore, mass-derived condition is indicative of intrinsic condition in juveniles of this system.

Juvenile condition was positively correlated with adulthood body mass in the Seychelles warbler, independently of permanent mass constraints such as sex and structural size. This indicates that between-individual differences in the variable component of juvenile mass (e.g. fat, muscle) is partially maintained across an individual's lifetime. Similar within-individual consistencies between juvenile and adult condition have been observed in other bird species (Merilä and Svensson, 1997; Guillemain *et al.*, 2013). Previous studies on adult Seychelles warblers have shown that mass is lost during energy-demanding reproductive behaviours (Komdeur, 2001; van de Crommenacker, Komdeur and Richardson, 2011; Bebbington *et al.*, 2017). Therefore, heavier juveniles may be

better able to maintain or recover lost energy reserves (i.e. mass) in adult-life, perhaps contributing to heavier juveniles also having higher rates of annual survival observed in this study. Conversely, achieving high juvenile condition at the expense of other physiological components could have negative consequences for adult condition. One potential trade-off a is greater rate of telomere shortening in early-life, resulting in shorter telomere lengths in adulthood (Monaghan and Ozanne, 2018). In many systems, including the Seychelles warbler, short telomeres and/or greater telomere shorting in adulthood also reflects more stressful life-histories and reduced survival prospects (Barrett *et al.*, 2013; Monaghan and Ozanne, 2018; Wilbourn *et al.*, 2018; Hammers *et al.*, 2019). However, we found no association between juvenile condition and adult telomere length, which suggests that the initial benefit of high juvenile condition does not have long-term physiological costs, at least when measured with telomere length.

We found that the survival benefits associated with high juvenile condition were not limited to the first year of life in the Seychelles warbler, with heavier juveniles also having higher annual survival throughout adulthood. This is consistent with our adult body mass analysis, which showed that individuals in better condition survived to older ages. Silver-spoon effects of early-life environment on adult survival have been observed in many wild populations (Reid *et al.*, 2003; Van De Pol *et al.*, 2006; Cartwright *et al.*, 2014; Alberts, 2019). Such effects may occur because juveniles that are heavier and/or reared in better natal environments have a competitive advantage that leads to them occupying better quality habitat as adults (Verhulst, Perrins and Riddington, 1997; Both, Visser and Verboven, 1999; Van De Pol *et al.*, 2006). Similarly, juveniles that survive to adulthood despite poor-natal environments, and hence poor condition, may have required compensatory physiological mechanisms that have delayed survival costs (Metcalfe and Monaghan, 2001; Briga *et al.*, 2017).

The silver-spoon effect of juvenile condition also contributes to lifetime reproductive success, since this is strongly correlated with longevity in this species (Davies *et al*. 2021). However, juvenile condition did not affect the probability of producing offspring in a given year (after controlling for age-effects), which is in contrast to studies that have measured the effect of the natal-environment on reproductive success (e.g. Nussey *et al.*, 2007; Douhard *et al.*, 2014). In this system, individual breeding attempts are strongly constrained by population density (i.e. limited availability of breeding positions) and seasonal food availability (Komdeur, 1992, 1996c). Additionally, the success of breeding attempts is likely to depend on fine-scale environmental variation, which was not accounted for in this study. Therefore, ecological constraints and confounds may limit the detectable influence of juvenile condition on annual reproductive success. Furthermore, the strong decline of annual reproduction in the terminal year likely means that poor-condition and/or illness in the current year outweighs the effect of past condition (Hammers *et al.*, 2012).

The effect of juvenile condition on annual survival was constant with age, and did not affect the onset or rate of survival senescence. This is consistent with a recent meta-analysis that found that the quality of early-life environments was not associated with survival senescence across 18 wild populations (Cooper and Kruuk, 2018). One explanation is that the majority of individuals that experience poor early-life conditions, or are themselves in poor condition, die before reaching senescent age (the age at which a population exhibits reduced survival), while the few individuals that reach old age share traits that mask the effects of early-life factors ("selection hypothesis"; Nol and Smith, 1987; Dugdale *et al.*, 2011). Another possibility is that the silver-spoon effect of juvenile condition is not associated with early-life investments (e.g. growth, reproductive effort) that have delayed costs for late-life performance (Hunt et al., 2004; Spagopoulou et al., 2020). For example, Hammers *et al*. 2013 identified in this species a trade-off between early-life reproductive effort and late-life survival; individuals that start breeding at earlier ages had an earlier onset of survival senescence. In contrast, our findings suggest that investments in early adult-life (in terms of age-specific annual reproduction) are not associated with juvenile condition. Therefore, juvenile condition may fail to generate such resource allocation trade-offs (i.e. between early-life reproductive effort and somatic maintenance) that influence senescence patterns.

4.5.1. Conclusion

Our study shows that a juvenile's mass-derived condition can be a marker of persistent individual differences in adult condition and performance. This finding reinforces the hypothesis that natal-environmental factors that influence juvenile mass can have individual fitness consequences beyond juvenile survival. While juvenile mass was not found to predict individual differences in senescence rates, either directly or via associations with early-life investments, juvenile condition is positively associated with longevity, and thus the likelihood of reaching the age at which senescence occurs in the population.

4.6. References

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4.7. Supplements

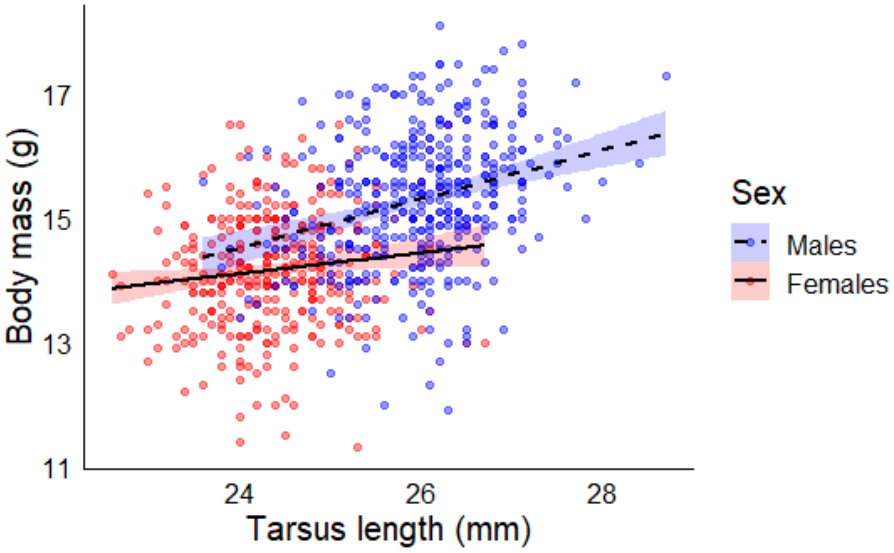

Figure S4.1; the relationship between body mass, tarsus length and sex for juvenile Seychelles warblers

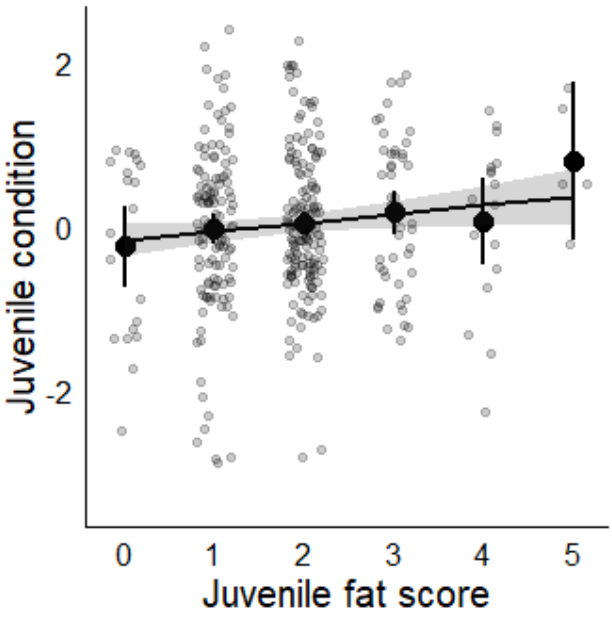

Figure S4.2; the relationship between juvenile condition and fat score for juvenile Seychelles warblers

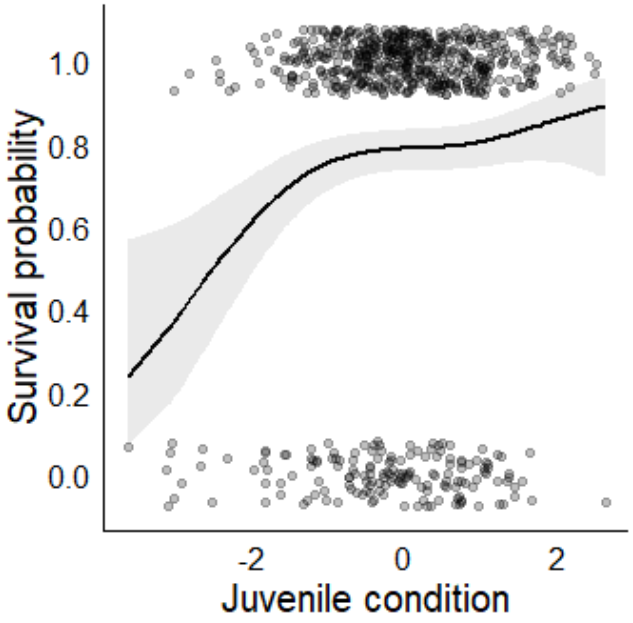

Figure S4.3: The probability of juvenile Seychelles warblers surviving to adulthood (>1 year of age) relative to their juvenile condition. The fit line is a nonparametric smoothing function (from GAMM analysis) between survival (Y/N) and condition with 95% confidence limits. Raw data points depict the distribution of jittered observed survival counts (1 = survived, 0 = deceased).

Table S4.1; The relationship between body mass, tarsus length, sex and catch time in juvenile Seychelles warbler. Condition (i.e. residual mass) was calculated by regressing body mass against tarsus length and catch time in separate models for males and females (due to the significant interaction between sex and tarsus length – see Fig. S1).

Predictor	**Estimate**	**SE**	***t***	***P***
(Intercept)	4.692	1.650	2.844	0.005
Tarsus length	**0.398**	**0.063**	**6.292**	**<0.001**
Sex (female)	**4.859**	**2.417**	**2.010**	**0.045**
Catch time	**0.001**	**0.000**	**3.327**	**0.001**
Tarsus length × Sex (female)	**-0.220**	**0.096**	**-2.277**	**0.023**
Random	**762 observations**		**Variance**	
Observers	39 observers		0.035	
Residual			0.886	

Table S4.2; The relationship juvenile survival, condition, sex and genetic variation in the Seychelles warbler.

Juvenile survival; conditional R^2 = 0.087				
Predictor	**Estimate**	**SE**	***z***	***P***
(Intercept)	0.819	1.072	0.764	0.445
Condition	**0.334**	**0.152**	**2.199**	**0.028**
Age (months)	0.803	2.003	0.401	0.689
Sex (female)	-0.250	0.306	-0.818	0.414
Tarsus length	0.193	0.165	1.175	0.240
Heterozygosity	-0.925	0.699	-1.324	0.186
$TLR3^A$ (no)	0.381	0.446	0.856	0.392
Ase-ua4 (no)	0.005	0.385	0.012	0.991
MHC diversity	1.093	0.877	1.247	0.213
Random	**235 individuals**	**Variance**		
Catch year	10 years	0.060		

Chapter 5
Epigenetic markers of chronological age and survival in a wild passerine

Seychelles warbler chick

5.1. Abstract

Within-populations there can be considerable individual variation in the onset and rate of senescence; individuals may have biological ages (BA) that differ from their chronological age (CA). Measuring CA and BA in wild populations is difficult but vital if we are to understand the complex social, environmental and genetic factors affecting senescence. DNA-methylation clocks, which generate age-predictions based on age-correlated methylation levels at cytosine-guanine pairs, offer a promising solution; accurately reflecting both CA and BA in humans. However, DNA-methylation clocks in wild populations remain taxonomically biased (towards mammals) and rarely incorporate BA-components, such as condition or survival. In this study, we developed ribosomal DNA-methylation clocks to estimate both CA and survival (a component of BA) in a population of Seychelles warblers (*Acrocephalus sechellensis*). Clock estimates of CA (derived from methylation at 67 CpG sites) were strongly correlated with known CA, with a mean difference of 2.2 years (based on leave-one-out cross-validation). Additionally, clock-estimated CA was influenced by terminal year effects; individuals sampled in their last year of life had a different pattern of methylation resulting in significantly lower clock-estimated CA, relative to known CA. Clock-estimated CA also increased within-individuals between sampling events; thus, rDNA-methylation exhibited longitudinal change with CA. Clock-estimates of survival to the next year (based on methylation at six CpG sites) were more accurate than, and independent of, other survival markers; body mass and telomere length. Therefore, our study highlights the utility of DNA-methylation clocks for measuring CA and BA in non-mammalian wild populations.

5.2. Introduction

Senescence – the age-related decline in performance and fitness – is widespread in vertebrate taxa (Jones *et al.*, 2013; Nussey *et al.*, 2013; Gaillard and Lemaître, 2020). Typically, as individuals grow older they reach an age from which their physiological performance and somatic integrity progressively declines. At the demographic-level, individual senescence results in lower reproductive success and/or survival in older cohorts (Nussey *et al.*, 2013; Hammers *et al.*, 2015; Lemaître and Gaillard, 2017). However, within- and between- populations of the same species there can be considerable variation in the onset and rate of senescence, meaning individuals may be 'biologically' older or younger (i.e. have lower or higher functional ability) than expected for their chronological age (hereafter CA; Nussey *et al.*, 2008; Gunn *et al.*, 2009; Bouwhuis *et al.*, 2010). Since CA does not necessarily reflect an individual's biological age (hereafter BA), measures of both CA and BA are essential for our understanding within disciplines concerned with organismal health and Darwinian fitness.

A long-standing goal in ecology and evolution is to understand why individuals senesce differently. The majority of current insight originates from studies of lab-based and captive model organisms;

crucially, identifying mechanisms which modulate the ageing process (Partridge, 2010; López-Otín *et al.*, 2013). However, such populations lack the variation and complexity – in terms of genetic background, life-history and environmental exposure – of natural populations. Therefore, findings from laboratory/captive environments cannot be generalized to natural populations (e.g. Briga and Verhulst, 2015). Longitudinal studies of wild populations, whereby individuals are monitored throughout their lives, offer the opportunity to test the applicability of lab-based findings in real-world settings, where individual fitness and senescence are realized under evolutionary relevant conditions (Nussey *et al.*, 2008). Wild populations are also uniquely suited for exploring the role of complex environmental and social factors on senescence patterns (Nussey *et al.*, 2007; Hammers *et al.*, 2015). For example, there is increasing awareness of the contribution cooperative breeding has in modulating ageing mechanisms and late-life survival (Berger *et al.*, 2018; Hammers *et al.*, 2019).

Determining CA in wild populations is fundamental for characterizing senescence and BA (i.e. relative health/condition with age cohorts). Age structure is also a key determinant of population dynamics, which is widely implicated in matters of conservation and sustainability (Iannelli and Milner, 2017); for example, the resilience of fish stocks to harvesting (Campana, 2001). However, the study of ageing in wild populations is often hindered by difficulties in measuring the CA of individuals (Jarman *et al.*, 2015), since many species do not possess external features from which CA can be accurately determined – such as tooth wear in deer (Pérez-Barbería *et al.*, 2014). The alternative strategy of marking young individuals in wild populations – so that the same individual can be aged at later dates – is often not practical, or extremely labour intensive and costly. Furthermore, non-model organisms lack the multitude of clinical health markers (i.e. available to human medicine) to characterise BA (e.g. Levine, 2013).

Molecular markers of ageing hallmarks can provide measures of both CA and BA (Jarman *et al.*, 2015; Jylhävä, Pedersen and Hägg, 2017). For example, telomere length – a commonly utilized age marker in wild population studies - has provided considerable insight into drivers of differential-senescence (Barrett *et al.*, 2013; Sudyka *et al.*, 2014; Young, 2018). However, telomere length has also proven difficult to reliably quantify – owing to measurement error in qPCR procedures (Verhulst *et al.*, 2013; Bateson, Eisenberg and Nettle, 2018) – and can be highly variable within-individuals. For example, individual telomere length can both decrease and increase in response to a range of factors (e.g. food availability, infection), independently of age and senescence (Hoelzl *et al.*, 2016; Asghar *et al.*, 2018; Brown *et al.*, 2021). Thus, telomere length does not provide accurate single measures of CA or BA (Dunshea *et al.*, 2011; Boonekamp *et al.*, 2013).

DNA-methylation is the binding of methyl groups (5-mC) to cytosine-guanine pairs (CpGs; Jones *et al.*, 2015). In humans, 70-80% of CpGs are methylated and, by silencing gene transcription, play an

essential in regulating gene expression (Bird, 2002). Methylation at certain CpGs can provide accurate estimates of CA (Horvath and Raj, 2018). The approach involves machine-learning methods to incorporate age-informative CpGs (typically from hundreds of thousands of CpGs across the genome) into regression models to derive an age prediction (Hannum *et al.*, 2013; Horvath, 2013; Levine *et al.*, 2018). For example, the Horvath clock, utilises 353 genome-wide CpGs to predict human age to within 3 years (Horvath, 2013). DNA-methylation clocks have been developed from several tissue types (e.g. blood, skin) in several wild mammalian taxa, including; cetaceans (e.g. Polanowski *et al.*, 2014; Robeck *et al.*, 2021), bats (Wilkinson *et al.*, 2021), monkeys (e.g. Anderson *et al.*, 2021; Horvath *et al.*, 2021), canids (e.g. Thompson *et al.*, 2017) and rodents (Horvath *et al.*, no date; Stubbs *et al.*, 2017). However, there remains considerable uncertainty regarding the general applicability of DNA-methylation clocks to wild animal ageing (see below).

The rapid expansion of mammalian DNA-methylation clocks is in large part due to consistent age-associated methylation at conserved CpGs (Polanowski *et al.*, 2014; Lu *et al.*, 2021) - meaning the same subset of CpGs can be screened across mammals at a relatively low cost, i.e. compared to whole-genome sequencing (Arneson *et al.*, 2021). Unfortunately, these conserved GpGs (used in mammalian DNA-methylation clocks) do not appear to exhibit similar age-associations in other taxa e.g. birds (De Paoli-Iseppi *et al.*, 2017). Thus far, the few DNA-methylation clocks developed outside of mammals have relied on whole-genome (where available e.g. chickens, *Gallus gallus domesticus*; Raddatz *et al.*, 2021) or reduced-representation approaches (e.g. short-tailed shearwaters, *Ardenna tenuirostris*; De Paoli- Iseppi et al., 2019). Alternatively, DNA-methylation clocks have been developed by targeting specific genes thought to be relevant to the ageing process (e.g. European bass, *Dicentrarchus labrax*: Anastasiadi and Piferrer, 2020). Ribosomal DNA – a small (ca. 5000 bp) highly-conserved gene region – based methylation clocks have been developed both in mammals (Wang and Lemos, 2019) and, recently, the European lobster (*Homarus gammarus*; Fairfield *et al.*, 2021), which suggests evolutionary-conserved DNA-methylation clocks are not restricted to mammals. A significant advantage of sequencing small gene regions (such as rDNA) is the substantial reduction in cost i.e. compared to whole-genome sequencing. That said, there remains a strong taxonomic bias in the development of DNA-methylation clocks.

There are several lines of evidence to suggest that characteristics of DNA-methylation can also reflect biological ageing in humans. Differences between clock-predicted and known CA inevitably occur due to individual-level variation in DNA-methylation. This difference – termed “epigenetic age acceleration” (EAA) – has been attributed to differences in BA and is linked to differences in survival prospects. A higher clock-predicted age, relative to CA (i.e. positive EAA), is associated with poorer cognitive and physical performance, age-related diseases, higher mortality risk, and life-style factors associated with faster ageing e.g. smoking (for review see Horvath and Raj, 2018). Interestingly, the rate of epigenetic ageing relative to known age (often referred to as “tick rate”)

is also non-linear – accelerated in adolescence and slower in elderly-life (Horvath, 2013; Bell *et al.*, 2019; Marioni *et al.*, 2019) – and is thought to reflect differing biological ageing throughout the life course (Simpkin *et al.*, 2017; Binder *et al.*, 2018). Therefore, DNA-methylation clocks (trained to predict CA) can offer dual functionality as both CA and BA markers (Bell *et al.*, 2019). However, improving the accuracy of CA estimates (i.e. reducing EAA to near-zero) inevitably limits the capacity of DNA-methylation clocks to also reflect BA (Zhang *et al.*, 2019). Hence, for direct estimates of BA (instead of CA) several DNA-methylation clocks have been developed incorporating age-related health markers and/or mortality as training data (Zhang *et al.*, 2017; Levine *et al.*, 2018; Lu *et al.*, 2019).

There is evidence that DNA-methylation clocks can reflect BA outside of humans. Interventions that extend lifespan and/or decelerate biological ageing (e.g. dietary restriction) are associated with lower epigenetic age (i.e. negative EAA) in captive mice and macaques (Maegawa *et al.*, 2017; Petkovich *et al.*, 2017; Stubbs *et al.*, 2017; Wang *et al.*, 2017). However, similar studies on wild populations are scarce, and mainly concern reproductive- and sex differences in epigenetic age (but see Pinho *et al.*, 2021). In both roe deer (*Capreolus capreolus*) and yellow baboons (*Papio cynocephalus*) adult males exhibit higher epigenetic ages (relative to females of the same age) and, in yellow baboons, male epigenetic age increases with social rank - and thus reproductive activity (Anderson *et al.*, 2021; Lemaître *et al.*, 2021). Similarly, the epigenetic age of castrated male sheep is lower than intact males, suggesting that DNA-methylation clocks reflect the costs of male reproduction on ageing and lifespan (Sugrue *et al.*, 2021). However, the epigenetic age of yellow baboons was not influenced by early-life adversity or social integration – the strongest predictors of lifespan in that system – and no study of a wild population has yet assessed whether epigenetic age, or DNA-methylation generally, is directly associated with survival or lifespan.

The isolated Seychelles warbler (*Acrocephalus sechellensis*) population on Cousin Island provides an excellent model system for the development and application of DNA-methylation clocks in a wild population. This system benefits from over 30 years of continuous monitoring of known-age individuals that have been captured and blood sampled repeatedly across their life-time (Komdeur, 1992; Hammers *et al.*, 2015; Sparks *et al.*, 2020). Furthermore, because of a lack of migration in or out of the population (Komdeur *et al.*, 2004), survival is accurately known without being confounded by dispersal (Komdeur, 1992; Richardson et al., 2007; Hammers et al., 2019). Here we assess correlations between CA and CpG-methylation at the rDNA gene region in the Seychelles warbler. We focused on rDNA as it is thought to harbour an evolutionary-conserved methylation-clock of both CA and BA (Wang and Lemos, 2019). Furthermore, due to the small size (*ca* 5 kbp) and abundance (≥300 copies per genome) of rDNA, methylation can be quantified via targeted sequencing at a relatively low cost. We developed an rDNA-methylation clock for estimating CA of individuals using cross-validated machine learning methods. We then determined whether

variation in EAA (i.e. clock-estimated age relative to known-age) is explained by sex, birth cohort and or terminal-year effects (whether or not the individual is in its last year of life). Terminal-year individuals are expected to be in poorer health/condition than individuals not in their terminal year irrespective of age (Hammers *et al.*, 2012); thus, we were interested in whether this difference was reflected in epigenetic age. Furthermore, using longitudinal samples we tested whether the rDNA-methylation clock accurately predicts within-individual differences in CA. Finally, using the same cross-validated machine learning methods as for the CA clock, we developed an rDNA-methylation "survival" clock to predict survival to the next year. We compared the predictive performance of this clock to that of survival models incorporating measures previously used in the Seychelles warbler, e.g. body condition and telomere length, to assess whether rDNA-methylation offers an improvement on alternative survival markers frequently applied to wild populations.

5.3. Methods

5.3.1. Study System

The Seychelles warbler is a small insectivorous passerine endemic to the Seychelles. The population on Cousin Island (29 ha; 4°20' S, 55°40' E) – containing *ca.* 320 adult individuals (Brouwer et al 2009) – has been extensively monitored since 1986 (Komdeur, 1992; Richardson, Burke and Komdeur, 2003; Hammers *et al.*, 2015; Sparks *et al.*, 2020). Since 1997, nearly all individuals (>96%) have been ringed with a unique combination of a British Trust for Ornithology (BTO) metal ring and three colour rings for identification (Richardson *et al.*, 2001; Raj Pant *et al.*, 2020). The population is structured into ca. 115 clearly defined territories (Kingma et al., 2016). Each year, during the major and minor breeding season (June-September and January-March, respectively) each territory is visited at least every two weeks to identify all individuals present and determine their status through behavioural observations (Richardson, Burke and Komdeur, 2003). The resighting probability of individuals during the major breeding season is close to one (0.98 for individuals ≥2 years-old (Brouwer et al., 2006)) and dispersal from the island is virtually absent (Komdeur et al., 2004). This provides extremely accurate estimates of survival, since individuals that are not observed during the major breeding season can be confidently assumed dead. Individual death is confirmed by a lack of resightings in the following breeding season(s).

5.3.2. Sampling procedure and selection

Individuals are usually first caught as nestlings, or as dependent juveniles (<5 months old) on their natal territory using mist nets (see Kingma *et al.*, 2016 for details). Juveniles are aged as fledglings (1-3 months), old fledglings (3–5 months) or sub-adults (5–12 months) based on behaviour and eye colour (Komdeur, 1992). In addition to capturing unringed juveniles, as much of the ringed adult population as possible (normally ca. 35%) is re-captured and sampled during both breeding seasons each year. For every capture event, ca. 25 ul of blood is taken by brachial venipuncture and stored

in 100% ethanol. Body mass is measured using either a Pesola spring balance or electronic scale (± 0.1g) and structural size is measured using sliding callipers (± 0.1 mm) as the length of the right tarsus. DNA extracted from blood samples was used to confirm sex (following Richardson et al., 2001). Relative Telomere Length (hereafter RTL; the concentration of amplified telomeric DNA relative to that amplified at GAPDH – a single copy gene) has also been measured as part of a previous study (for details see Spurgin et al., 2017 and Chapter 3).

From our extensive longitudinal database, we selected samples from individuals from four birth cohorts (2002, 2005, 2006 and 2010) for DNA-methylation analysis. We included all individuals sampled at ca. 1-year-of-age (± 6 months) as a reference point (hereafter 'first sample') from which rDNA-methylation could be compared to older ages within the individual's lifetime i.e., longitudinal change. We excluded samples from chicks and young fledglings since age-dependent DNA-methylation during growth has been shown to differ considerably from that of adults (Horvath, 2013). Where multiple later samples were available for an individual, we excluded those less than one year apart. Where >3 samples per individual were available, we selected the three samples most widely distributed within the individual's lifetime i.e. the first, last and most equidistant middle sample. We purposefully retained individuals which only had a first sample (i.e., died before being recaptured) so not to bias our findings towards only long-lived individuals.

5.3.3. DNA-extraction and rDNA alignment

Genomic DNA was extracted from a c. 2 mm^2 flake of whole blood using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. DNA concentration and purity were quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific). Extracted DNA was stored at -20°C in low Te buffer. Ribosomal DNA occurs in tandemly repeated clusters which range from 8 to 14 kb in animals (Dyomin *et al.*, 2016). Each rDNA copy comprises three genes (18S, 5.8S and 28S), internal transcribed spacers and external transcribed spaces (5'ETS and 3'ETS). Using a partially constructed genome, we identified the Seychelles warbler rDNA cluster via alignment to the zebra finch (*Taeniopygia guttata*) reference genome using BLASTn in NCBI. One scaffold was found to contain all three rDNA genes (Accession numbers: XR_004366572.2, XR_004366574.1, XR_004366570.1) which had extremely high cross-species similarity to that of the zebra finch (96.8%, 100% and 100% identical, respectively).

5.3.4. Bisulphite Sequencing

Targeted bisulphite sequencing including primer design, validation and bioinformatics was conducted by Zymo Research. Primers were created using Rosefinch (Zymo's proprietary sodium bisulfite converted DNA-specific primer design tool) such that PCR amplicons would be between 100-300 bp and avoid annealing to CpGs. Where not possible, primers were synthesized with a pyrimidine (C/T) at the CpG cytosine in the forward primer, or a purine (A/G) in the reverse primer

to minimize amplification bias to either the methylated or unmethylated allele. Primers were tested using Real-Time PCR with 1 ng of bisulfite-converted control DNA (from one sample), in duplicate individual reactions. DNA melt analysis was performed to confirm the presence of a specific PCR product. PCRs were deemed successful if the following criteria were met; average crossing point (Cp) values of <45, duplicate Cps have a Cp difference of <1, melting curves in the expected range for PCR products, and duplicate melts with calculated melting-temperatures within 10% of the coefficient of variation. Following primer validation, ca. 500 ng of DNA per sample was bisulfite converted using the EZ DNA Methylation-Lightning™ Kit (Zymo Research). Multiplex amplification of all DNA samples was performed using Fluidigm Access Array™ System. The resulting amplicons were pooled for barcoding according to guidelines outlined in Fluidigm Access Array specification. After barcoding, pooled amplicons were purified (DNA Clean & Concentrator-5™) and prepared for massively parallel sequencing using a MiSeq V2 300 bp Reagent Kit (Illumina) and paired-end sequencing protocol.

Sequence reads were identified using standard Illumina base-calling software and analyzed using a Zymo Research proprietary analysis pipeline. Sequence reads were aligned back to the reference genome using Bismark, an aligner optimized for bisulfite sequence data and methylation calling (Krueger & Andrews, Bioinformatics, 2011). The proportional methylation scores (0–1) for a given CpG were estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T.

5.3.5. Data filtering

Methylation scores from read depths of <50 were converted to NAs (not applicable). GpGs with >10% NA scores across samples were excluded, while those with <10% NA scores had NAs replaced with their mean methylation score, thus ensuring potentially informative GpGs are not excluded based on missing scores. GpGs that tended to always be methylated or un-methylated across samples (i.e., <10% and >90% methylated) were also excluded.

For our training dataset (see below) we assessed the effect of sample storage time (present date – blood sampling date) on CpG methylation and mean rDNA-methylation per sample, while controlling for individual age (samples < 1.5 years-of-age). A section of 17 consecutive CpGs in 28s exhibited moderate correlations (Pearson's r > 0.4) with sample storage time (present date – blood sampling date). Since sample storage time and individual age are to some extent collinear (i.e., samples from older individuals were taken more recently), we removed these 17 CpGs from methylation clock models. Mean rDNA-methylation per sample was not correlated with sample storage time.

5.3.6. Development of chronological-age clock

For training our rDNA-methylation clock we selected only one sample per individual to ensure that model accuracy and validation were not biased with consistent between-individual differences in rDNA-methylation. The age-distribution of samples in the full dataset was heavily right-skewed (i.e., more first samples were available than re-captures), which has been shown to reduce the predictive performance of DNA-methylation clocks (Mayne, Berry and Jarman, 2021). This was also the case in our data; clocks trained on the full dataset (including longitudinal samples) had considerably poorer predictive performance than the final clock (results not reported). Therefore, we selected the oldest available sample per individual to improve the evenness of sample age-distribution and maximize the representation of old ages.

All analyses were performed in Rstudio (v1.2.5033 and R v4.0.3, Rstudio Team, 2020). To determine how correlated methylation at each CpG was with age, we performed multiple Pearsons correlation tests. All CpGs were included as predictors of CA in penalized regression models using glmnet (v4.1-1; Friedman, Hastie and Tibshirani, 2010). Within penalized regression, two parameters are adjusted for model performance, (i) lambda; the stringency of penalty (high lambdas = stronger penalization) and, (ii) alpha; the elastic-net mixing parameter, which for our analyses was set to 1 or 0.5 (equivalent to LASSO and elastic net-regression, respectively). The optimal lambda value (i.e., the level of penalization that produces the least root mean square error (RMSE) in age estimates; *lambda.min*) was calculated following 10-fold cross-validation in cv.glmnet. Since the 10-folds within cv.glmnet are selected at random, *lambda.min* changes with each iteration of cv.glmnet. To reduce variability, we use mean *lambda.min* following repeated runs (100x) of cv.glment. All GpGs that passed the mean *lambda.min* threshold were incorporated into the model.

5.3.7. Evaluation and validation of chronological age clock

The resulting models were used to predict the CA of each sample. To evaluate how well each model fitted the data, we calculated the mean absolute difference (MAD) between predicted- and known-age, and R^2 from a linear regression between predicted- and known-age. However, assessing model accuracy based on models fitted to the training dataset may result in biased estimates due to overfitting. To achieve unbiased estimates of model accuracy, and assess how well the models are likely to perform on unknown data, we performed a Leave-One-Out-Cross-Validation (LOOCV) in Caret (v6.0-86; Kuhn, 2020). During LOOCV, all samples but a single “test” (i.e., left-out) sample are included in the penalized regression (i.e. model training), with the resulting model being used to predict the age of the omitted sample. This process is repeated for every sample i.e., each is omitted from model training once. The caret package automatically identifies the ‘best’ model (i.e. that which produces the least RMSE in omitted samples) from a range of lambda values predefined in

cv.glmnet. MAD and R^2 between predicted- and known-age (for test samples), were calculated as for the full dataset.

5.3.8. Other determinants of clock-predicted age

Predicted-age can deviate from known-age due to individual differences in methylation at CpGs included in the DNA-methylation clock, which can be associated with life-history, environmental exposure and condition (Horvath and Raj, 2018). In linear regression models, we determined whether variation in predicted-age (derived from the full and LOO-CV versions of the 'best' age model) can be explained by sex, cohort year or terminal year effects (defined as whether or not the individual was alive one year after the sample event; yes/no). By controlling for known-age (fixed effect), predicted-age (response) can be interpreted as Epigenetic Age Acceleration (EAA). Using an F-test we also compared the level of variance in EAA (i.e. residual variance) between terminal year groups (yes/no).

5.3.9. Longitudinal change in predicted age

DNA-methylation clocks trained on cross-sectional age may be confounded by consistent between-individual differences in quality and/or lifespan i.e., selective disappearance. We used the 'best' rDNA-methylation clock to predict the age of individuals (used for model training) at earlier sample events (which were omitted from model training). Our objective was not to predict the age of these earlier samples *per se* but rather to assess whether within-individual changes in predicted age correspond to the known increase in age between sampling events, thus determining the models ability to predict longitudinal ageing. We performed a linear mixed model (LMM) in *lme4* (v1.1-25; Bates *et al.*, 2015) with the difference between predicted-age across consecutive samples (hereafter "Δpredicted-age") as the response variable, while the known-age difference between consecutive samples (hereafter "Δknown-age") was included as a predictor. We also included the starting known age (i.e., age at the initial sampling event) as a predictor, since Δpredicted-age may vary with age; for example, if predicted ageing is accelerated in old individuals due to senescence. Also included in the model as predictors were sex, birth cohort year, terminal year (yes/no) and individual identity as a random factor; since individuals with three samples had two measures of Δpredicted-age.

5.3.10. Development of survival-clock

Using a separate DNA-methylation clock, we determined whether differential rDNA-methylation can be used to predict survival prospects; whether or not the individual died within a year after sampling (yes/no). For this analysis, we included only the last available sample per individual since, by definition, individuals always survived >1 year after earlier sample events. To identify CpGs that are informative of survival we used the penalized regression (LASSO) in glmnet. As for the CA clock, 100 iterations of 10-fold cross validation were used to select *lambda.min;* the level of penalization

that produces the least misclassification error (AUC). For the final model we examined the overall classification accuracy, as well as individual associations between methylation and survival for GpGs retained in the final model.

We were interested in whether methylation at survival-associated CpGs (those with non-zero coefficients in the LASSO regression) was independent of other factors linked with survival (e.g. age). For a subset of samples, data were also available for body condition (body mass controlling for skeletal body size, i.e., tarsus length) and relative telomere length (RTL) at the time of sampling; both of which have been associated with survival in Seychelles warblers in previous studies based on larger datasets (Barrett *et al.*, 2013, Brown *et al*., 2021). In linear regression models, we tested whether methylation at survival-linked CpGs was associated with age, sex, body mass, tarsus length and RTL. For samples with complete data, we also compared logistic regression models (with survival as the response) with different combinations of predictors. These models included a *base* model (with body mass, tarsus length, telomere length, sex and age as predictors), a *CpG marker* model (with CpGs selected by the LASSO-regression as predictors) and a *base + CpG marker* model (all predictors). Model predictive power and fit were compared using receiver operating characteristic (ROC) curves and Akaike Information Criterion (AIC), respectively.

5.4. Results

5.4.1. Development of chronological age clock

A total of 527 rDNA CpGs were incorporated into our analyses. Of these 29 (5.5%) exhibited methylation that was significantly correlated with known CA across our model training dataset (n =

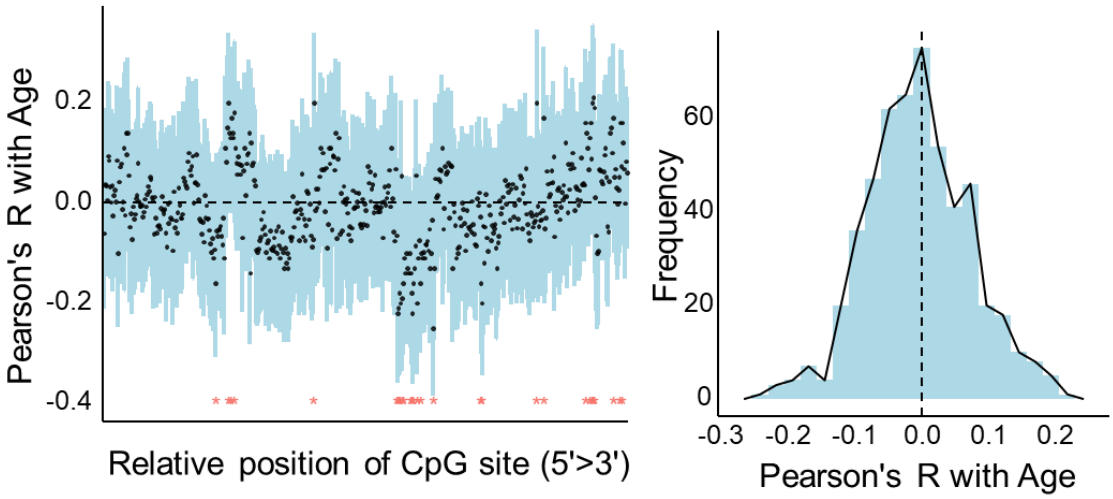

Fig. 5.1: Pearson's correlations between percentage methylation and chronological age for 527 CpGs in the 18s, 28s, and 5.8s rDNA genes for Seychelles warbler samples included in our DNA-methylation clock training dataset (*n* = 166). Red * in left panel denotes significant correlations (*P* < 0.05).

166, Pearson's R, P < 0.05, Fig. 5.1). Comparing the performance of the two penalized-regression models investigated (Elastic-net and LASSO), using MAD and R^2, both models had near-identical performance. However, we selected the LASSO for the final model as it tended to utilise ca. 10 fewer CpGs (57/527) than the Elastic-net, so only the LASSO results are shown here. These CpGs were evenly distributed across 18s, 28s and 5.8s, and exhibited both increasing and decreasing methylation with age.

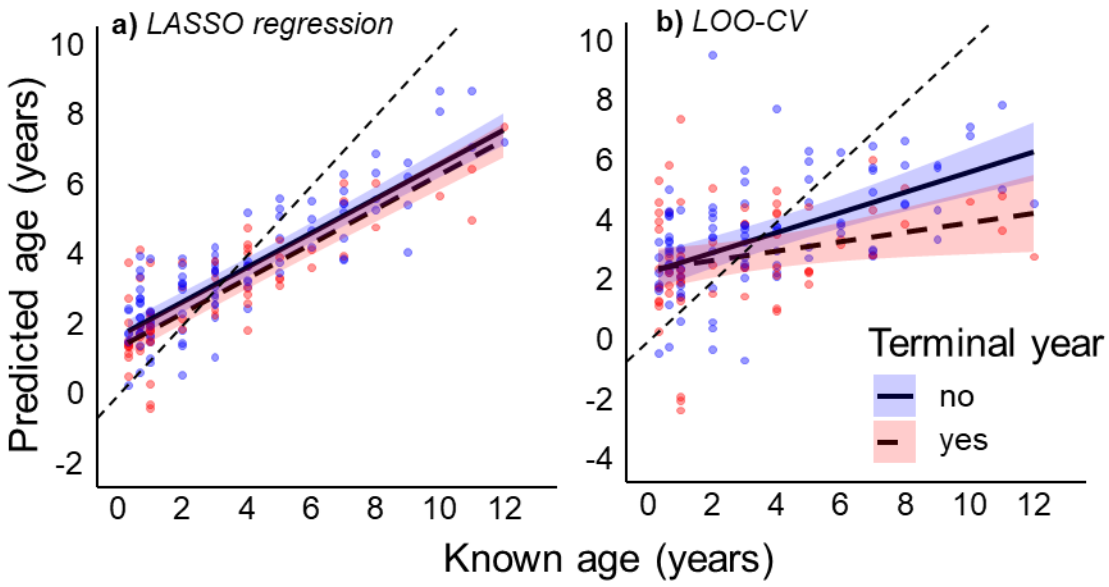

Figure 5.2: The relationship between methylation clock-predicted age (derived from a LASSO regression) and known chronological age of Seychelles warblers. Panel a) displays model performance on the full training dataset, while b) displays model performance following Leave-One-Out Cross-Validation (LOO-CV). Solid lines are linear regression predicted slopes with 95% CI for samples taken within one year of the individual's death (terminal year = yes, in orange) and not within one year of the individual's death (terminal year = no, in blue). Diagonal dashed lines denote the perfect relationship (y = x). Points are raw data grouped by terminal year.

5.4.2. Evaluation and validation of chronological age clock

According to the LASSO regression, predicted age was strongly correlated with known CA, explaining 73% of the variation (R^2 = 0.73; Fig. 5.2). Known CA was estimated to within 1.4 years by predicted age (MAD = 1.1 years). In comparison to the LASSO regression (which utilized the full training dataset), the LOO-CV model (which was run using the same parameters as the LASSO regression) selected 67 CpGs. The predictive accuracy for omitted samples (i.e., those left-out of model training) was substantially lower (R^2 = 0.21, MAD = 2.19; Fig. 5.2). With both the LASSO regression and LOOCV models, there was a tendency to over-estimate young ages by ca. 2 years (y-intercept of Fig. 5.2) and under-estimate old ages.

5.4.3. Other determinants of clock-predicted age

Predicted ages (from the LASSO and LOO-CV models) were not influenced by sex (Table 5.1). There was a significant global effect of cohort on LASSO predicted age. However, this effect was weak, as Tukey pair-wise comparisons revealed no significant differences between the four cohorts. Predicted ages were lower for individuals in their terminal year, relative to individuals of the same CA not in their terminal year (Table 5.1; Fig. 5.2). For the LOO-CV analysis, the difference in predicted age between terminal and non-terminal individuals increased with CA (Table 5.1; Fig. 5.2). Residual variance did not significant differ between terminal year groups (LASSO-predicted age; F = 0.833, P = 0.410, LOO-CV-predicted age; *F* = 0.796, *P* = 0.305).

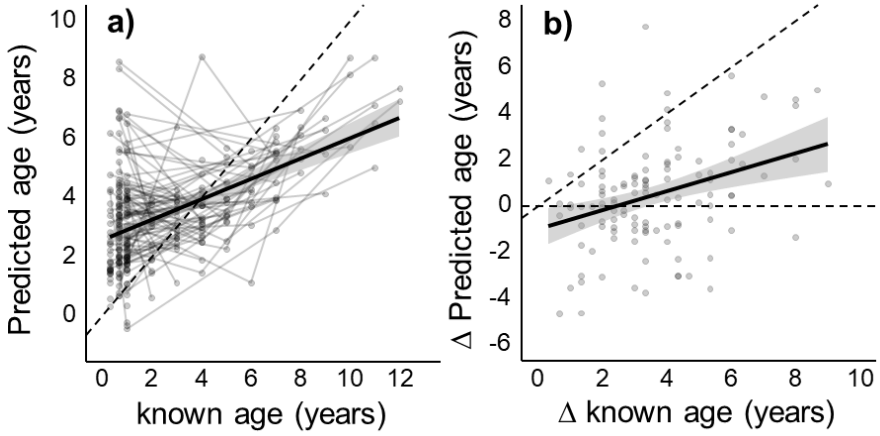

Figure 5.3: The relationship between methylation clock-predicted age (derived from a LASSO regression) and known age of Seychelles warblers – including longitudinal samples omitted from model training. Panel a) shows predicted age versus known age for the full dataset, with thin grey lines connecting multiple samples from the same individual. Panel b) shows the change in predict age versus change in known age, for individuals with two or more samples. Solid lines are linear regression predicted slopes with 95% CI. Diagonal dashed lines denote the perfect relationship (y = x) and the horizontal dashed line of b) marks the zero intercept. Points are raw data.

5.4.2. Longitudinal change in predicted age

We used the LASSO-regression to estimate the age of repeat samples (originally omitted from model training) from the same individuals (see methods; Fig. 5.3a) to calculate Δpredicted-age and Δknown-age. Results show that Δpredicted-age was strongly correlated with Δknown-age (Table 5.2, Fig. 5.3b). However, Δknown-age was consistently under-estimated i.e., the within-individual change in estimated age was less than the known increase in age (Fig. 5.3b). Furthermore, there were several incidences where the earlier sampling event was incorrectly assigned as the older of the two samples, meaning sample pairs exhibited a negative change in predicted age (Fig. 5.3b; points under y-intercept line). Negative-Δpredicted-age was more frequent when the duration between sample events was short (low Δknown-age) and from sample pairs where the first

sampling event occurred at ca. 1 year-of-age, as predicted-age of ca. 1 year-olds was often substantially over-estimated (Fig. 5.3a). In contrast, nearly all (17/19) sample pairs in middle age (starting age ≥3 years) exhibited an increase in Δpredicted-age.

Table 5.1: Linear regression models explaining variation in methylation-clock predicted age of Seychelles warblers. Cohort year refers to the year in which individuals are born (reference cohort year is 2002).

LASSO predicted age				
	Estimate	SE	*t*	*P*
(Intercept)	1.696	0.191	8.885	< 0.001
Age	**0.495**	**0.024**	**20.879**	**< 0.001**
Terminal year (yes)	**-0.312**	**0.146**	**-2.145**	**0.034**
Sex (male)	0.012	0.142	0.083	0.934
Cohort year (2005)	0.104	0.193	0.539	0.591
Cohort year (2006)	-0.113	0.204	-0.552	0.582
Cohort year (2010)	**0.392**	**0.190**	**2.062**	**0.041**
Age × terminal year (yes)	-0.085	0.048	-1.765	0.079
LOO-CV predicted age				
	Estimate	SE	*t*	*P*
(Intercept)	2.330	0.359	6.492	< 0.001
Age	**0.338**	**0.053**	**6.329**	**< 0.001**
Terminal year (yes)	0.111	0.388	0.286	0.775
Sex (male)	-0.025	0.255	-0.098	0.922
Cohort year (2005)	0.127	0.347	0.366	0.715
Cohort year (2006)	-0.435	0.367	-1.184	0.238
Cohort year (2010)	0.586	0.342	1.713	0.089
Age × Terminal year (yes)	**-0.180**	**0.087**	**-2.065**	**0.041**

5.4.5. Survival

Six of the 527 CpGs were selected by the LASSO-regression as predictors of survival over the next year. Methylation at four of these CpGs was positively associated with survival, while two were negatively associated (Fig. 5.4). Only one of these CpGs (1866 position of 28s) was incorporated in both the CA- and survival- LASSO regressions. The model had an overall classification accuracy of 70% i.e., the model correctly classified 70% of samples as originating from an individual that died or survived the subsequent year. Of the 166 samples included in model training, 120 also had both body condition and RTL data. Methylation at the six survival-associated CpGs was not associated with body condition nor RTL (Table S5.1); however, methylation on CpG (436 position of 28s) was positively associated with age (Table S5.1). A logistic regression model using the six CpG markers (as predictors of survival) performed significantly better than models that included only body

condition and RTL, along with other controls; age, sex and tarsus length (Fig. 5.5). The model with all predictors (CpG markers + based model) had the highest discriminatory power (AUC = 0.84) but did not have a better model fit than that of the CpG marker-only model (ΔAIC < 4).

Table 5.2: Linear mixed model explaining variation in Δmethylation-clock predicted age between consecutive blood samples (longitudinal sampling) from Seychelles warblers. Cohort year refers to the year in which individuals are born.

	Estimate	**SE**	***t***	***P***
(Intercept)	-1.261	0.588	-2.145	0.034
Age	**0.613**	**0.122**	**5.009**	**<0.001**
ΔAge	**0.333**	**0.098**	**3.382**	**0.001**
Sex (male)	0.071	0.366	0.193	0.847
Cohort year (2005)	0.113	0.489	0.232	0.817
Cohort year (2006)	-0.182	0.558	-0.327	0.744
Cohort year (2010)	-0.501	0.463	-1.081	0.282
Terminal year(yes)	-0.268	0.391	-0.686	0.494
Random	**117 observations**		**Variance**	
Individual identity	98 individuals		<0.001	

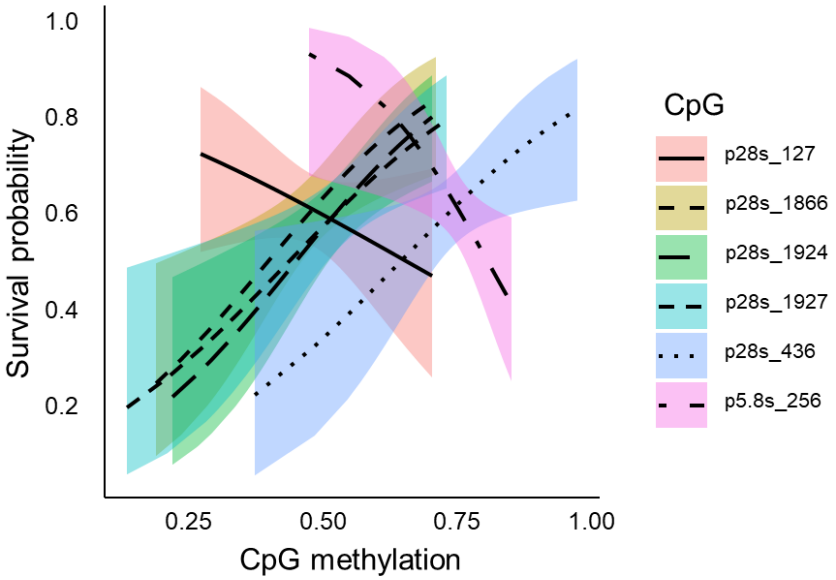

Figure 5.4: The probability of survival (alive >1-year post sampling; Y/N) in Seychelles warblers relative to percentage methylation at six CpGs with non-zero LASSO-regression coefficients. Fit lines are binomial regressions between survival and methylation with 95% confidence limits.

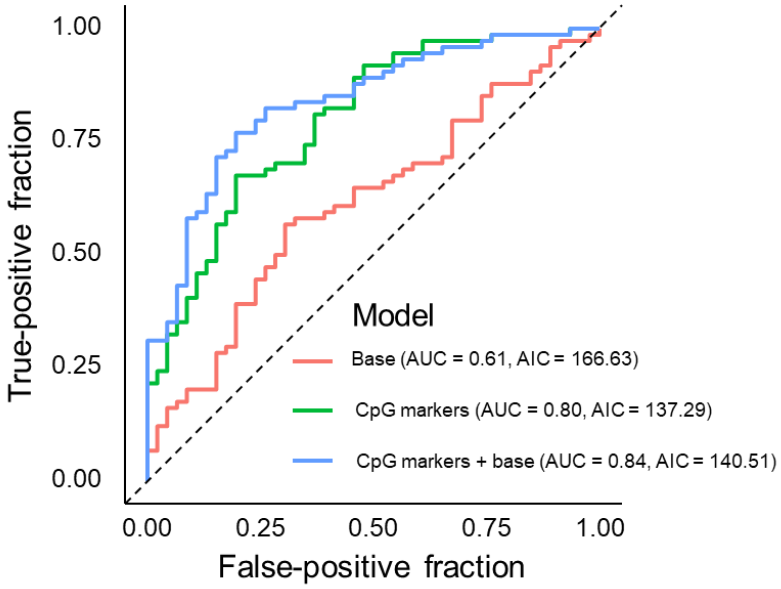

Figure 5.5: Receiver operating characteristic (ROC) curves for the overall performance of logistic regression models to predict survival (alive >1-year post sampling; Y/N) of Seychelles warblers. The base model contained age, sex, body mass, tarsus length and telomere length as predictors. The CpG marker model contained six CpGs with non-zero LASSO-regression coefficients. The Area Under the ROC Curve (AUC) is a measure of discriminatory power; 1 = maximum discrimination, 0.5 = no discrimination (denoted by dashed diagonal line). AIC is Akaike Information Criterion.

5.5. Discussion

Methylation of rDNA in whole-blood samples was used to develop a CA clock in Seychelles warblers. Predicted-age was strongly correlated with the known CA of individuals in our model training data set; however, the correlation between predicted- and known-age was weaker (though still significant) for test individuals omitted from model training. Interestingly, EAA (epigenetic age relative to known age) differed in relation to the individual's survival prospects – individuals in their terminal year had lower EAA than individuals not in their terminal year – in both training and test individuals – irrespective of CA. The methylation-clock – when used to predict CA of individuals at earlier sampling events – under-estimated longitudinal ageing and tended over-estimate the age of ca. 1 year-olds. A separate model was developed using rDNA-methylation to predict survival (1-year post-sampling; Y/N) using the same training dataset. Methylation at CpGs selected by this "survival-clock" were better at predicting survival than (and independent of) conventional survival markers i.e., body condition, RTL and CA.

The strong correlation between rDNA-methylation clock predicted age and known CA of the training dataset was similar to that seen for other DNA-methylation clocks in other species (e.g. De Paoli-Iseppi *et al.*, 2019; Anderson *et al.*, 2021; Lemaître *et al.*, 2021). There was also a tendency to over-

estimate the age of young individuals and under-estimate the age of old individuals, which is a common feature of DNA-methylation clocks (Anderson *et al.*, 2021; Horvath *et al.*, 2021), and penalised-regressions more generally (Engebretsen and Bohlin, 2019). However, our clock had relatively poor predictive performance when applied to test data using the leave-one-out-sample approach (i.e., low precision) compared to many other studies, which suggests measures of fit from the training dataset were biased by model overfitting. This may have been caused by the age-diminishing sample size of our training dataset – despite selecting the oldest available sample per individual - since CpGs were selected (via penalised regression) based on their fit to comparatively few old-age samples (Mayne *et al.*, 2021). Furthermore, relationships between DNA-methylation and older ages can differ from that of young-to-mid ages (i.e. due to survivor- and/or ceiling-effects), which may further bias models fitted to fewer old-age samples (Marioni *et al.*, 2019).

The lower leave-one-out-sample precision was most likely caused by the underlying correlations observed between CA and CpG-methylation in our data, which were less numerous and weaker compared to the age-correlations identified in other studies (Polanowski *et al.*, 2014; De Paoli-Iseppi *et al.*, 2019; Lemaître *et al.*, 2021), including those specific to rDNA; in mammals (Wang and Lemos, 2019) and the European lobster (Fairfield, 2021). In some cases, age can be accurately estimated using only a restricted subset of the strongest age-correlated CpGs (Weidner *et al.*, 2014; Han *et al.*, 2018). Due to the ultra-conserved nature of rDNA, it seems unlikely (albeit possible) that a lack of strong age-correlations in the Seychelles warbler rDNA reflects taxonomic differences between birds, mammals and crustaceans. Our contrasting findings could reflect different rDNA-methylation patterns in erythrocytes - compared to lymphocytes (Wang and Lemos, 2019) and leg/claw tissue (Fairfield, 2021) – as tissue-specificity has been observed in human DNA-methylation clocks (e.g. Horvath, 2013). Nevertheless, our rDNA-methylation clock could offer an improved means of estimating individual age in systems which lack alternative methods. Such is the case for birds, since external features (e.g. plumage moults) can only discriminate board age categories (e.g. juvenile versus adult; Rohwer, 1996) and RTL (the only other widely-applied molecular marker) is generally weakly associated with age (Pauliny *et al.*, 2006; Salomons *et al.*, 2009; Spurgin *et al.*, 2018, but see Haussmann and Vleck, 2002).

The low precision of our DNA-methylation CA clock compared to other studies may also reflect differences in internal cross-validation procedure between studies. In our analytical pipeline, test data (i.e., left-out samples) were not included in model regularization/optimization (i.e., the selection of CpGs via penalised regression on training data) to ensure complete independence of training and test data (as suggested by e.g., Horvarth 2013). However, in some studies (e.g. Polanowski *et al.*, 2014; De Paoli-Iseppi *et al.*, 2019), full datasets (including samples subsequently used as "test" data) were used for model optimization/CpG selection, which could lead to overly optimistic estimates of out-of-sample precision. Additionally, in De Paoli-Iseppi *et al.*, 2019, age was

predicted from simple multiple linear regression (incorporating CpGs with non-zero coefficients from LASSO-regression) not penalized regression, which could lead to overfitting since this removes coefficient shrinkage penalties (Engebretsen and Bohlin, 2019). To illustrate, adopting similar methodology as in those studies we achieve higher out-of-sample precision in our data (R^2 = 0.52, MAD = 1.7 years; not reported in results due to the aforementioned concerns). More generally, studies have utilized many different methods for both building and validating DNA-methylation clocks, often with little justification for the method chosen, and a consensus on best practice has yet to be reached.

Strong age-correlations of rDNA-methylation may have been lacking in our system due to confounding effects of other factors. In contrast to Wang and Lemos, (2019), and Fairfield *et al.*, (2021), which utilized mainly captive individuals for training their rDNA-methylation clocks, our wild population is subject to more complex and varied environments that could generate more variation in rDNA-methylation, and thus clock-predicted ages. For example, deviations in clock-predicted ages in humans are often associated with health parameters (e.g. diet, stress) and associated lifestyle factors - presumably because these factors modulate methylation scores at CpGs in DNA-methylation clocks (Horvath and Raj, 2018; Noroozi *et al.*, 2021). Similarly, rDNA-methylation is also modulated by dietary interventions that extend lifespan in captive rodents (Wang and Lemos, 2019; Gensous *et al.*, 2020). Therefore, deviations between clock-predicted and known age might reveal important biological differences between-individuals.

Interestingly, we found that Seychelles warblers in their terminal year exhibited significantly different methylation patterns, having lower predicted ages compared to individuals not in their terminal year. Furthermore, this difference was more pronounced in (chronologically) older individuals. The direction of this effect contrasted with that of human studies, whereby a lower predicted age (relative to known age) is associated with better health and survival prospects, i.e., low BA (Marioni, *et al.*, 2015; Marioni, *et al.*, 2015). Therefore, low predicted age does not seem to reflect lower BA in terminal-year Seychelles warblers, but could reflect differences in health/survival that are age-independent. For example, studies have shown that terminal-year individuals can have lower reproductive success (Coulson and Fairweather, 2001; Rattiste, 2004; Hammers *et al.*, 2012) or maximise reproductive effort at the expense of condition (i.e. terminal investment Clutton-Brock, 1984; Velando, Drummond and Torres, 2006; Fisher and Blomberg, 2011) and thus are expected to be in poorer condition and/or more stressed than individuals not in their terminal year. In our Seychelles warbler population, individual condition and stress levels are highly variable due to, for example, differences in territory quality, competition, infection and breeding-stage (Van de Crommenacker *et al.*, 2011; van de Crommenacker, Komdeur and Richardson, 2011; Bebbington *et al.*, 2017; Spurgin *et al.*, 2018). Therefore, the observed terminal-year-effect suggests deviations in clock-predicted age (from known age) could reflect individual-

differences in health/condition at the time of sampling. Identifying drivers of variation in clock-predicted age is crucial for the development of future DNA-methylation clocks in wild populations, not only for achieving more accurate CA estimates, but also for exploring the utility of DNA-methylation as markers of other biological traits, such as relative condition.

Relationships between traits and cross-sectional age can reflect consistent between-individual differences associated with lifespan, such as individual quality, rather than within-individual changes in the trait (Nussey *et al.*, 2008). This is especially true for wild populations, since often only a few disproportionately high-quality individuals survive to old age, i.e., selective disappearance occurs (e.g. Hayward *et al.*, 2013). Therefore, previous studies have (where available) used longitudinal data to assess DNA-methylation clocks abilities to track within-individual chronological aging (De Paoli-Iseppi *et al.*, 2019; Anderson *et al.*, 2021; Lemaître *et al.*, 2021). Consistent with these studies, we found that Δpredicted-age and Δknown-age were positively correlated, meaning an increase in an individual's known-age was associated with an increase in predicted age. Therefore, rDNA-methylation - at CpGs from which predicted-age is derived - exhibits longitudinal change with age. As with cross-sectional age, there was a tendency to underestimate Δknown-age, which is also observed in humans (e.g. Marioni *et al.*, 2019). There was also a high occurrence of negative Δpredicted-age (i.e., predicted-age was relatively higher when the individual was chronologically younger), in large part due to the rDNA-methylation clock overestimating age of ca. 1 year-olds omitted from model training. Why this occurred is unclear but could reflect statistical artefacts – such as regression-to-the-mean – or differences in age-distributions between cross-sectional and longitudinal datasets. Alternatively, rDNA-methylation may be more variable and/or change more rapidly in young individuals – for example, due to differing rates of maturation (Horvath, 2013; Binder *et al.*, 2018; Lemaître *et al.*, 2021) - making CA harder to estimate. However, with more longitudinal DNA-methylation data, our system is well-suited for determining whether rates of epigenetic ageing vary during the life-course or in response to life-history events.

We found the CpG methylation at six CpGs could predict survival to the next year. Methylation at these CpGs was not associated with body condition or RTL and only one CpG was associated with CA - which have been linked with Seychelles warbler survival in previous studies (Barrett *et al.*, 2013; Brown *et al.*, 2021) - which indicates that these CpGs are independently associated with survival. Furthermore, survival models based on the six CpGs outperformed survival models based on RTL, body condition and CA on the same dataset. These findings are similar to that of human studies comparing DNA-methylation clocks and RTL; DNA-methylation clock estimates are independent of RTL and/or more strongly associated with mortality and health than RTL (Breitling *et al.*, 2016; Marioni *et al.*, 2016; Chen *et al.*, 2017). The reasons for these differences remain

uncertain and are beyond the scope of this study but point towards different pathways/mechanisms of individual health being reflected by rDNA-methylation and telomeres.

Differing rDNA-methylation could be causally implicated in mortality and biological ageing by causing the dysregulation of nucleolar activity (proposed by Wang and Lemos, 2019). However, much like RTL (Young, 2018), the majority of evidence concerning associations between DNA-methylation and mortality is correlative i.e. DNA-methylation is associated with third-party factors (e.g. disease, life-style) that cause differing survival (Bell *et al.*, 2019). Therefore, methylation at survival-linked CpGs could be used both as a marker of relative health/condition, and for identifying drivers of differential survival and (potentially) actuarial senescence – which could be pursued with a larger sample size of old individuals (onset of actuarial senescence in this population is ca. 7 years; Hammers *et al.*, 2013). Such investigations would also benefit from more longitudinal data to assess whether methylation at survival-linked CpGs reflects consistent individual-differences in longevity, or whether methylation at survival-linked CpGs changes within-individuals with CA and/or prior to death.

5.5.1. Conclusion

In conclusion, we demonstrate that rDNA-methylation can reflect aspects of both CA and BA in a wild population. Achieving more accurate estimates of CA may require a larger and more evenly age-distributed sample size, or the incorporation of methylation assays across other candidate genomic regions. A significant novel finding is that epigenetic CA estimates can reflect individual-differences in survival prospects in a wild population, and thus this should be a consideration for developing more accurate CA-clocks in the future. Our study highlights a promising application of DNA-methylation as a new and improved marker of survival prospects, which could greatly benefit the study of ageing and senescence in wild populations.

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5.7. Supplements

Table S5.1: Linear regression models explaining variation in rDNA-methylation at six survival-linked CpGs in the Seychelles warbler (n = 120). RTL is relative telomere length.

p28s_127	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.453	0.169	2.675	0.009
Body mass	0.006	0.007	0.871	0.385
Age	-0.001	0.003	-0.539	0.591
RTL	-0.026	0.034	-0.761	0.448
Tarsus length	-0.001	0.008	-0.174	0.862
p28s_436	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.777	0.191	4.068	0.000
Body mass	-0.006	0.007	-0.860	0.392
Age	**0.007**	**0.003**	**2.316**	**0.022**
RTL	0.025	0.039	0.641	0.523
Tarsus length	0.001	0.009	0.100	0.921
p28s_1924	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.123	0.218	0.563	0.574
Body mass	-0.007	0.009	-0.819	0.415
Age	0.005	0.003	1.316	0.191
RTL	0.039	0.044	0.879	0.381
Tarsus length	0.019	0.010	1.848	0.067
p28s_1927	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.094	0.217	0.432	0.667
Body mass	-0.003	0.008	-0.407	0.685
Age	0.006	0.003	1.724	0.088
RTL	0.049	0.044	1.125	0.263
Tarsus length	0.017	0.010	1.733	0.086
p28s_1866	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.397	0.204	1.943	0.055
Body mass	0.000	0.008	0.002	0.999
Age	-0.003	0.003	-0.834	0.406
RTL	-0.015	0.041	-0.365	0.716
Tarsus length	0.004	0.009	0.451	0.653
p5.8s_256	**Estimate**	**SE**	***t***	***P***
(Intercept)	0.708	0.131	5.412	0.000
Body mass	-0.002	0.005	-0.396	0.693
Age	0.001	0.002	0.336	0.737
RTL	0.040	0.027	1.490	0.139

Tarsus length	0.001	0.006	0.222	0.825

Chapter 6
Discussion

Giant Aldabra tortious taking a nap on Cousin

One of the overall objectives of this thesis was to explore the utility of different intrinsic markers for measuring individual-level variation in physiological senescence in the Seychelles warbler (SW). In doing so, these markers could offer a valuable means of quantifying the costs of life-history factors to somatic maintenance, and their consequences for fitness/performance in late-life. In Chapter 2, I report a complex relationship between haematocrit, age and age-specific survival. In Chapter 3, I identify several life-history factors that influence bidirectional telomere dynamics (in a sex-specific manner) and the association between those dynamics and future mortality risk. In Chapter 4, I reveal a silver-spoon effect of juvenile condition on adult condition and annual survival, but no effect of juvenile condition on late-life performance. Finally, in Chapter 5 I utilise ribosomal DNA (rDNA)-methylation sequence data (generated specifically for this project) to develop a DNA-methylation clock for predicting chronological age and survival, which I then show considerably improves on the resolution provided by previous markers (e.g., telomere length).

The advantages and disadvantages of each specific intrinsic marker (for the study of ageing and senescence) are discussed in detail in their respective chapters. Thus, to avoid repetition in this present chapter, I focus more on synthesising findings from across chapters. Firstly, I explain how my findings reaffirms, and build upon, previously reported patterns of senescence in the SW. Secondly, I discuss how and why the intrinsic markers investigated in this study might exhibit similar and/or contrasting associations with age, fitness, and life-history in the SW, and how the use of multiple intrinsic markers might improve our understanding of senescence in the SW and other systems. Lastly, I determine which single intrinsic marker investigated in this study offers the most promising means of studying senescence in wild populations, and practical considerations for applying this intrinsic marker to other systems. Throughout, I discuss broader recurring themes that have emerged from the findings of this project. I also address the limitations and uncertainties regarding my findings, and I propose solutions/directions for how these may be resolved in future research.

Before the start of my project, the senescence (or lack thereof) of demographic traits in the SW (i.e. annual survival and reproduction) and some intrinsic markers (e.g. oxidative stress, telomere length, malaria prevalence) had already been well-documented, revealing an asynchrony in SW ageing (Hammers *et al.*, 2015); as observed for various other traits in several other wild systems (e.g. Hayward *et al.*, 2015). Several of my findings reaffirm these patterns with the inclusion of more recent data. For example, in Chapter 5 I report similar patterns of actuarial and reproductive senescence, and terminal-year effects on reproductive success, as reported in Hammers *et al.*, 2012 and 2013. At the time of those earlier publications, age-specific reproductive success of females was based on observational data (i.e. the presence/absence of fledglings) which can be confounded by co-breeding and breeding attempts outside of the main breeding seasons. However, my findings confirm these earlier observations with the use of the now available genetic pedigree, in which the

genetic parentage for >85% of the caught population is determined with high confidence (Edwards, Burke and Dugdale, 2017). Additionally, my findings have revealed new patterns of change in key intrinsic markers used previously in the SW system; for example, the associations between telomere dynamics, malaria infection, and reproductive effort (Chapter 3). Such findings can offer a means of measuring previously hidden costs of life-history factors on late-life performance (but see below).

The findings of my project have added to the observed asynchrony in SW ageing, i.e., the varying onset and rate of change in fitness-related traits with age. For example, haematocrit declines within-individuals from ca. two years-of-age; around five years before the onset on actuarial senescence (Hammers *et al.*, 2013). By contrast, telomere length shortens non-linearly with age; the rate of shortening is greatest in the first year-of-life (Spurgin *et al.*, 2018) after which telomere length changes relatively little with age (i.e. minor shortening) in adult-life. Body mass exhibits a similar relationship but in reverse - increasing rapidly in the first year-of-life but exhibiting no consistent within-individual change with age in adult-life. Different age-associations among traits suggests they reflect mechanisms/processes with differing contributions for senescence. For example, we did not observe deleterious effects of low haematocrit levels from two years-of-age, which suggests declining haematocrit is not a senescent trait *per se* (discussed in Chapter 2). Traits that do not change with age, but are associated with fitness, likely reflect consistent individual differences in quality (and thus longevity) that resist senescent declines (Cohen *et al.*, 2020). Such seems to be the case for body condition in the SW. Similarly, telomere length, although associated with age, is better explained by largely age-independent factors. These observations pose important questions for future research in the SW and other systems. To what extent are age-dependent changes in traits correlated within individuals? Are combinations of several traits, or interactions among traits, more informative of late-life performance than a single-marker approach? Do traits respond differently and/or antagonistically to life-history factors? For example, do individuals maintain body mass in the face of environmental stress at the cost of telomere length, or vice versa?

Senescence-related traits in the SW such as annual survival and telomere length, while associated with age, are also highly variable. For example, at the population-level, annual survival decreases from *ca* seven years-of-age and the average lifespan is ca 5.5 years (Hammers *et al.*, 2015). However, some individuals live more than three times longer than the average lifespan (Hammers and Brouwer, 2017). The extent to which variation in survivability or telomere length reflects differential senescence is unclear, since variation in these traits can also represent differences in individual quality and short-term environmental effects. For example, it was recently shown that variation at the Toll-Like Receptor 3 gene (a viral-sensing gene) is associated with survival and lifespan in the SW (Davies *et al.*, 2021). Similarly, In Chapter 4 I found that some variation in annual

survival, independent of age, is attributed to differences in juvenile mass. Hence, variation in annual survival/longevity may be better explained by constraint-based mechanisms (i.e., genetics and silver-spoon effects) rather than differential senescence.

In Chapter 3 I demonstrate that some of the high within-individual variability in telomere length reflects short-term differences in life-history stresses, such as malaria infection. As discussed in Chapter 3, this finding suggests that telomere dynamics poorly reflect differential senescence in the SW; instead, telomere length/dynamics may be better interpreted as a condition/stress marker (but see below). Similar conclusions may be drawn regarding haematocrit and adult body mass/condition, since both traits are associated SW survival but are also highly variable within-individuals. For example, haematocrit and body mass vary with respect to reproductive status/stage in the SW (Chapter 2; Komdeur, 2001; van de Crommenacker, Komdeur and Richardson, 2011; Bebbington *et al.*, 2017). Therefore, these markers could have utility as a measure of life-history costs to current or short-term condition/survival, e.g., reproduction-survival trade-offs. However, the lack of strong relationships with age (at least in later-life) combined with high temporal variability (which may mask associations with long-term performance) means that these intrinsic markers are poor-direct measures of senescence.

Due to the inherent limitations of correlative studies, a recurring uncertainty of this project is the extent to which intrinsic markers are causally implemented in biological ageing and/or mortality of SWs. Experimental studies, whereby survival/lifespan is compared between control and treatment groups (for which intrinsic markers are manipulated) can answer this question. For example, targeted interventions (e.g. drugs) for increasing haematocrit and telomere length are both available/feasible in birds (Williams *et al.*, 2012; Reichert *et al.*, 2014). However, implementing interventions that may be detrimental to individual health would conflict with other goals of the SW project, particularly the conservation of this rare species. Hence, the SW system is poorly suited for experimental investigations of this nature. That said, determining causality with experimental manipulation may prove difficult if targeted interventions have effects that are not limited to the intrinsic marker of interest. For example, artificially increasing telomerase activity (to increase/maintain telomere length) has restorative effects on cells that are independent of telomere length (Cong and Shay, 2008; as discussed in Chapter 3).

Nevertheless, whether causality can be determined does not preclude intrinsic markers from providing valuable insight. A purely correlative marker that reflects several causal ageing mechanisms (or their combined effect) may be more desirable, especially if the causal mechanisms are themselves difficult or unfeasible to measure directly. Indeed, DNA-methylation clocks are perhaps the best marker of human biological age not necessarily because of causality but because

DNA-methylation provides a proximal readout of a collection of molecular ageing mechanisms (discussed in Horvath and Raj, 2018).

A further limitation of this project's correlative findings is that the extent to which intrinsic markers (and by extension ageing and senescence) reflect life-history costs/stresses is uncertain, as this may be confounded by associations with intrinsic quality. Addressing this uncertainty is important for determining the relative contributions of early-late life history trade-offs (LHT) and constraints to senescence in the SW and other wild populations (Ardia, 2005; McLean, Archie and Alberts, 2019). This issue was discussed in relation to telomere dynamics, i.e., do dominant females exhibit telomere lengthening as a consequence of alleviated reproductive costs, or because these females (which have acquired high-quality territories and nest helpers) are intrinsically better?

The issue of early-late LHTs versus individual quality is likely to be relevant to follow-up investigations of other intrinsic markers. For example, based on the findings of Chapter 2, one might expect haematocrit to be positively associated with environmental stress in early-life. However, an equally valid hypothesis is that individuals with low haematocrit are better at avoiding environmental stresses, and thus have higher survival. Disentangling the relative contributions of life-history costs and individual quality is possible with experimental treatments aimed at modifying life-history costs. For example, food supplementation and experimentally reducing reproductive effort can result in telomere lengthening and/or reduced shortening; thus, supporting the theory that longitudinal telomere dynamics reflect early-late life-history trade-offs (discussed in Chapter 3). However, similar manipulations would be difficult or impossible to implement in the SW system. For instance, nests are rarely accessible for brood manipulations.

Differential methylation at ribosomal DNA (rDNA) is the most promising intrinsic marker of differential senescence so far tested in the SW. Even with limited data, rDNA-methylation was strongly associated with both age and survival in the SW, and outperformed existing measures, i.e., body condition and telomere length. Perhaps the most convincing support for our rDNA-methylation (trained to predict chronological age) is that it also detected a terminal-year effect, suggesting that deviation from the expected methylation-age relationship is a marker of poor-health, relative to age.

A practical advantage of DNA-methylation clocks over other age/senescence markers is their wider applicability to other wild systems. A key asset of long-term field studies, such as the SW system, is the extensive longitudinal and intergeneration datasets they accumulate over decades of monitoring. The majority of long-term field studies have routinely collected and stored DNA samples (e.g., whole blood) from the onset, whereas other sample types (e.g., blood plasma) may have been adopted more recently and/or intermittently; such is the case for the SW system. Therefore, a benefit of DNA-methylation markers over non-DNA-based markers (e.g., cytokines,

hormones – from blood plasma) is that they can be readily applied to the existing extensive datasets of long-term field studies. A further advantage of DNA-methylation clocks is that they are fine-tuned to existing data. The model training procedure is explained fully in Chapter 5, but briefly; DNA-methylation clocks utilise the most informative subset of CpGs (i.e., that best explains the target variable, e.g., age, health, survival etc.) from a much larger pool of CpGs (sometimes several thousand) in a given dataset. Therefore, DNA-methylation clocks are inherently more malleable to cross-species differences compared to single-trait markers such as haematocrit and telomere length, as evident by the rapidly growing number of species for which DNA-methylation clocks have been developed (at least for chronological age; Horvath and Raj, 2018; Bell *et al.*, 2019).

Further analyses (and data) are needed to determine whether DNA-methylation is specifically associated with late-life performance declines in the SW and other wild populations. A logical next step for our existing rDNA-methylation clock is to determine whether epigenetic age is associated with other components of late-life fitness, such as reproductive success, and factors thought to modulate senescence trajectories, e.g., early-life adversity. A further question is whether rDNA-methylation (or clock-predicted chronological age) is responsive to factors throughout the life-course, such is the case for telomere dynamics, e.g., does malaria infection make individuals epigenetically older? By design, DNA-methylation clocks of chronological age assume linear relationships between CpG-methylation and age. However, the rate of senescence is typically nonlinear with age; thus, CpGs with step-changes and/or accelerated changes in methylation in late-life (i.e., from the onset of actuarial senescence) might be better markers of senescence. For example, Johnson *et al.* (2017) showed in humans that methylation at two CpGs (residing in the promoter of a gene involved in immune cell differentiation) is relatively stable in early-life, but becomes progressively hyper-methylated and more variable from mid- to late-life, perhaps reflecting individual differences in immunosenescence. Such investigations are important to gauge the additional insight gained from DNA-methylation markers over existing markers (e.g., telomeres) in the study of senescence and life-history.

In conclusion, the extensive long-term dataset of the Seychelles warbler project provides a rare opportunity to explore the utility of several candidate intrinsic markers of senescence in a single wild population. Taken together, my findings show that intrinsic markers (and thus the somatic and physiological traits they represent) can exhibit varying associations with age, survival and life-history. Hence, this thesis emphasizes the importance of using multiple intrinsic markers, or at least avoiding an over-reliance on single markers, for a means of measuring the causes and fitness consequences of individual-level variation in senescence. More generally, this thesis opens the way for future research investigating the diversity and evolution of senescence patterns in nature.

6.1. References

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