**Strong cohort effects on early-life telomere length in a wild population**

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**Running head:** Early-life telomeres and survival

### Abstract

Understanding the short and long term costs of individual early-life experiences is fundamental to understanding life-history evolution. Telomeres, the protective caps at the ends of chromosomes, shorten in response to oxidative stress, and telomere shortening is correlated with reduced survival. Thus, it is possible that telomere dynamics may help us quantify individual variation in early-life costs, and enhance our understanding of how poor conditions in early life are related to later-life survival. We tested how telomere dynamics are related to spatio-temporal variation in early-life conditions and later-life survival in the Seychelles warbler (*Acrocephalus sechellensis*), using a large dataset of 935 juveniles from multiple cohorts spanning 16 years. Despite cross-sectional data showing that telomere length decreases with age, and most that telomere length decreases most rapidly early in life, using longitudinal data we found that significant reductions in telomere length can only be detected within the first few months of life - after this, longitudinal changes in telomere length were not significantly different from zero. Juvenile telomere length varied markedly among cohorts, and this cohort variation was related to the season (birds born in winter seasons had longer telomeres), and to population density (birds bird in years with higher populaiton density had longer telomered). Within cohorts, we found no evidence that the early-life social environment or ecological conditions were related to telomere length or rates of shortening. Finally, we found no relationship between early-life and survival to adulthood. Our data suggest that telomeres are subject to strong population-level variation, but suggest that the resolution of telomeres as a useful biomarker of individual-level cost is likely to be age-dependent, and in many cases limited.

**Keywords:** Life-history; Seychelles warbler; Senescence; Survival

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### Introduction

Exposure to favourable environmental conditions during development and growth can confer fitness advantages later in life (so called 'silver spoon effects'; Grafen 1988; Monaghan 2008). Adult fitness can be affected by a range of early-life experiences, including the quality of the habitat and available resources (Madsen and Shine 2000; Van de Pol et al. 2006; Hayward et al. 2013), population density (Nussey et al. 2007; Douhard et al. 2013) and natural or anthropogenic environmental disturbance (Reid et al. 2003; Cartwright et al. 2014). Understanding the causes and consequences of these early-life experiences is key to understanding many ecological and evolutionary processes, including patterns of natural and sexual selection, population growth rates and even local extinction (Coulson et al. 2001; Roach and Carey 2014). Understanding silver spoon effects is therefore of central interest to ecologists, evolutionary biologists and conservationists.

While it is clear that silver spoon effects can occur, we have little understanding of how and under what conditions early-life environments will affect adult fitness. We expect the later-life consequences of a good or poor start to vary among individuals, populations and species because the phenotypic consequences of an adverse environment can depend on an individual's initial condition, and genetic or epigenetic makeup (Hoffman and Hercus 2000; Richards 2006). Moreover, it is not always possible to fully quantify what constitutes a good or bad environment, and any 'hidden' environmental variation may obscure relationships between the early-life conditions that are measured and the resulting adult phenotypes. Indeed, the pervasiveness of silver spoon effects varies between species (Drummond et al. 2011), cohorts (Reid et al. 2003) and sexes (Wilkin and Sheldon 2009). We therefore need to understand, or at least be able to measure, how the environment affects individuals differentially within a population in order to elucidate the later-life consequences of early-life experience.

Telomeres - the protective caps on the ends of chromosomes - may provide a solution to this problem. Telomeres shorten with age (Monaghan and Haussmann 2006), and in response to oxidative stress, which can be elevated due to environmental factors (Von Zglinicki 2002). When telomeres become critically short cells senesce (Campisi 2003), and the accumulation of these cells can result in organismal senescence and death (Wong et al. 2003). This association between senescence and telomere length has inspired a great deal of recent research into telomere evolutionary ecology (reviewed in Horn et al. 2010; Haussmann and Marchetto 2010; Monaghan 2014). While there is little direct evidence that the relationship between telomere dynamics and survival is causal (Simons 2015), there is mounting evidence that telomeres can act as biomarkers of cost in wild populations, providing a signature of the ecological stress that has been experienced and is otherwise difficult to detect (Monaghan 2014; Schultner et al. 2014; Asghar et al. 2015).

There is evidence from a range of taxa that the greatest rate of telomere shortening occurs in early life (e.g. Frenck et al. 1998; Haussmann et al. 2003), and that the extent of this shortening is influenced by the conditions experienced during that period (Price et al. 2013; Monaghan 2014; Nettle et al. 2015b; Reichert et al. 2015). Early-life telomere dynamics have been associated with both short-term and late-life survival (Heidinger et al. 2012; Boonekamp et al. 2014), and with other parameters such as cognition (Nettle et al. 2015a). However, few studies have simultaneously analysed how telomere dynamics, early-life conditions and late-life survival are all related in a natural setting. Moreover, how early-life telomere dynamics vary over spatial and temporal scales is poorly understood.

The longitudinal study (since 1986) of the Seychelles warbler (*Acrocephalus sechellensis*) population on Cousin Island provides an excellent system for studying senescence in the wild (reviewed in Hammers et al. 2015). Due to the isolated nature of the study population and intensive field monitoring, we have unusually comprehensive survival data and tissue samples spanning many years (see Methods, below). Ecological conditions and warbler population density on Cousin vary across space and time due to weather-induced changes in foliage cover and food availability (Van de Crommenacker et al. 2011). Seychelles warblers remain on their natal territories for at least six months, and variation in the oxidative stress experienced by individuals is associated with natal territory quality (Van de Crommenacker et al. 2011). However, neither early-life nor adult survival appear to be associated with natal territory quality or natal local density (Brouwer et al. 2006; Hammers et al. 2013). Facultative cooperative breeding occurs in the Seychelles warbler (Komdeur 1994; Richardson et al. 2003b), and the presence of helping subordinates (but not non-helping subordinates) in the natal territory is associated with increased survival later in life (Brouwer et al. 2012). Lastly, we have an established protocol for assessing absolute telomere length in this species (Barrett et al. 2012), and telomere length predicts survival independently of age in adult Seychelles warblers, suggesting that telomeres act as a biomarker of cost (Barrett et al. 2013). Thus, we have an excellent system in which to assess the costs of different social and environmental conditions experienced early in life, and to assess the later-life consequences of early-life conditions.

In this study we examine how telomeres are related to early-life environmental variation and short and long-term survival in the Seychelles warbler. Because telomere dynamics are expected to reflect individual-level variation in the costs of early-life experiences, they may allow a more sensitive analysis of the effects of early-life environmental variation than would be possible with a direct comparison of how survival is affected by the early-life environment. With this in mind, we first conduct an exploratory analysis of how the environmental and social factors experienced in early life affect telomere dynamics. We then test the hypothesis that longer telomeres and lower rates of telomere shortening in early life are associated with greater survival.

### Methods

##### Study species and sampling

The Seychelles warbler is a small (~15 g), insectivorous passerine bird with a mean life expectancy of 5.5 years at fledging (Hammers et al. 2013). The population of *ca*. 320 birds on Cousin Island (04'20'S, 55'40'E) has been intensively studied since 1986 (Richardson et al. 2003a; Spurgin et al. 2014). This species has two breeding seasons, running from June-August (main breeding season) and December-February (minor breeding season), when the breeding females on each of the *ca*. 115 territories lay one or, rarely, two or three eggs (Komdeur et al. 1991). As a result of this low reproductive output, combined with higher mortality in first-year birds (39%; Brouwer et al. 2006), cohort sizes in the Seychelles warbler are small (<50).

Individuals are usually ringed in their first year of life, and so are of known age. They are then followed throughout their lives, and as they are non-migratory endemics naturally confined to the island (Komdeur et al. 2004), a biannual census of birds on Cousin during each breeding season gives accurate measures of local density, social status (e.g. breeding male/female, helping subordinate, non-helping subordinate) and individual survival (Crommenacker et al. 2011; Barrett et al. 2013). The isolated nature of the Cousin population is a key advantage of the system for analyses involving survival, which in other systems are often confounded by emigration (see Ergon and Gardner 2014 for a recent discussion). Full details of catching and monitoring methods can be found in Brouwer *et al.* (2012).

Seychelles warblers are highly territorial and all territories are mapped during the breeding seasons using detailed observational data of foraging and territorial defence behaviour, and surveyed for territory quality (Richardson et al. 2003a). Territory quality is calculated based on territory size, foliage cover and insect abundance (Komdeur 1992), and territory quality estimates obtained across years are averaged to obtain a single value for each territory (Hammers et al. 2013). Cousin is subject to intra- and inter-annual variation in rainfall and food availability, and such island-wide temporal variation may override the effects of individual territory quality. As an estimate of seasonal variation in food availability, we calculated an index of the number of insects across the entire island during each breeding season. This index is calculated as the mean number of insects found per unit leaf area over all surveys carried out on the island in a breeding season.

Each time a bird is caught on Cousin body mass and tarsus length are measured (to the nearest 0.1g and 0.1mm, respectively). Using information on eye colour (Komdeur 1991) and previous captures (Richardson et al. 2003a), we grouped birds into three age categories: one month old (birds still in the nest), 6 months old (fledglings with light grey eyes) and 10 months old (subadults with light brown eyes). A blood sample (*ca* 25 l) is taken from each bird captured via brachial venipuncture, and stored at room temperature in 1 ml of absolute ethanol in a 1.5 ml screw-cap microfuge tube.

##### Molecular methods

For each sample, genomic DNA was extracted from a ~2 mm2 flake of preserved blood using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol, with the modification of overnight lysis at 37oC and a final DNA elution volume of 80 l. Sex was determined using the PCR-based method outlined by Griffiths *et al.* (1998). Prior to telomere analysis, DNA concentration and purity were quantified using a NanoDrop 8000 Spectrophotometer (ThermoScientific), and the following thresholds were applied before samples were included for further analysis: i) DNA concentration must be at least 15 ng l-1 (based on a mean of three measurements), ii) the 260/280 ratio has to be between 1.8 and 2 and, iii) the 260/230 ratio should be higher than 1.8. DNA integrity was further validated by visualization with ethidium bromide after electrophoresis on a 1.2% agarose gel, and all samples with evidence of DNA degradation were re-extracted or excluded.

All DNA extractions that passed the above criteria were diluted to 3.3 ng l-1 before telomere measurement. We measured telomere length for all samples using a quantitative PCR (qPCR) assay of telomeres and a GAPDH control gene, using the molecular methods outlined by Barrett *et al.* (2012), with one amendment. A change in batch of SYBR green forced us to raise the annealing temperature of the telomere reaction from 58oC to 61oC for the majority of samples. However, this did not affect final telomere length values (see below). We used the program LinRegPCR (Ruijter et al. 2009) to correct for baseline fluorescence and calculate efficiencies and Cq values for each sample replicate. Averaging of technical repeats was carried out using custom-made R scripts (available as supplementary material), excluding samples with Cq values differing by >0.5. We then calculated relative telomere length (RTL) for each sample using equation 1 in Pfaffi *et al* (**???**). We chose to use RTL rather than continuing with the previously used method for calculating absolute telomere length (Barrett et al. 2012), as i) using RTL enabled us to run more samples per plate (as an oligo standard is not required), ii) the RTL method was less susceptible to batch effects (Appendix 1), and iii) very few other studies have calculated absolute telomere length, and our experience suggests that cross-species comparisons are unlikely to be reliable. Inter-plate repeatability of final telomere lengths was assessed using the R package rptR (Schielzeth and Nakagawa 2011), and for all subsequent analyses we used mean telomere length per sample where we had repeats.

For a subset of first-year birds for which we had longitudinal data, with an additional sample taken as an adult. For these individuals we calculated the within-individual change in RTL by subtracting adult RTL from early-life RTL (hereafter RTL). Negative values of RTL reflect telomere shortening, while positive values reflect telomere lengthening.

##### Statistical analyses

We performed all analyses using R version 3.0.1 (R Development Core Team 2011). Because we are using a different measure of telomere length to the previous study of adult telomere dynamics in this species (**???**), as well as including many additional samples, we first re-tested, using all available samples, whether telomere length decreases with age and age (a longitudinal measure based on within-subsject centring; Pol and Wright 2009) in the Seychelles warbler, using linear mixed models, with individual ID as a random effect. For the analysis of age we we only included birds who had been sampled at least twice. We then explored how telomere length varied within the first year of life (telomere length vs age in months) and over time (telomere length vs cohort ID). For this analysis we had bvery few birds with multiple samples, so we used linear models including only the first sample for each individual.

To assess our resolution for detecting telomere shortening, we tested, for each age class, whether RTL was significantly lower than zero, using one-sample t-tests. We then tested how RTL was related to the time interval between sampling events, using linear models. A significantly negative slope indicates that telomere shortening occurs linearly over time, while the time point at which RTL is significantly lower than zero can be considered the time period over which we have the resolution to detect reductions in telomere length.

We used general linear mixed models with model averaging to explore how spatial variation in early-life environmental and social conditions influenced telomere length within cohorts. Model averaging was carried out using the MuMIn package (version 1.10.5) in R (Bartoń 2012). We first created a full model containing the following explanatory variables: age (in months), tarsus length, sex, territory quality, season (summer or winter) and the number of helping and non-helping subordinate birds present in the natal territory. We also included interaction terms between age and all the other variables as telomere dynamics are expected to vary within the first year of life (Heidinger et al. 2012). As random effects we included cohort, territory ID and qPCR plate ID. Model selection was then performed and a top model set defined, containing all models with AICc 6 compared to the best supported model (Burnham et al. 2011). We report model-averaged coefficients, confidence intervals and 'relative importance', which reflects the relative weights of each predictor variable across the top model set. For individuals with longitudinal data (*n* = 340) we repeated the above analyses of telomere dynamics, replacing telomere length with telomere loss as the response variable, and excluding the plate ID random effect (as each measurement of telomere loss was based on two or more measurements, and so run on multiple plates).

We used generalised linear mixed models to test whether telomere length/loss were related to survival to adulthood. Models were set up with a binomial error structure, and included RTL/RTL and juvenile age as explanatory variable, along with the same random effects as for the model averaging (see above). We then used Cox regression, implemented in the survival package in R (**???**), to test whether long-term survival was related to individual-level telomere dynamics. For the cross-sectional data we used lifespan (in years) as survival time, while for the longitudinal data we used remaining lifespan from the second sampling event. We ran these models with cohort as a frailty term to exclude cohort effects, and only included cohorts in which at least 70 percent of individuals had died (all years up to and including 2007).

### Results

We measured telomere lengths using a total of 1686 unique samples from juvenile and adult Seychelles warblers. Of these, 935 samples were taken cross-sectionally from birds caught within their first year of life, and of these juvenile birds, we had additional longitudinal samples for 340. Efficiencies (mean ) for our telomere and GAPDH reactions were (1.80 0.04) and (1.93 0.04) respectively. Inter-plate repeatability of RTL, based on 392 samples measured at least twice, was 0.78 (CI = 0.73-0.82).

##### Early-life RTL

Considering the entire Seychelles warbler lifespan, RTL decreased with age (t = -7.31; P < 0.001; Fig. 1A), and with age (t = -3.82; P < 0.001; Fig. 1B). RTL also decreased with age within the first year of life (i.e. excluding adult samples; R2 = 0.03; t = -5.42; P < 0.001). Early-life RTL varied significantly among cohorts (one-way ANOVA, telomere length: F = 3.32; P < 0.001), and cohort-level variation in juvenile RTL (taken as the median for each cohort) was positively related to adult population density (R2 = 0.27; t = 3.04; P = 0.01; Fig. 1C). Variation in cohort-level RTL was not related temporal variation in territory quality (linear regression, R2 = 0.07; t = -1.33; P = 0.20), or island-wide food availability (R2 = 0.07; t = -1.30; P = 0.21).

The top model explaining variation in early-life RTL contained age, sex, season (summer vs winter), tarsus length, and the interaction between age and tarsus length (Table S1). The top model was much better supported than a null model (AICc = 36.55), and with the exception of the sex and tarsus effects, model-averaged confidence intervals did not overlap zero (Fig. 2A). RTL was higher in winter compared to summer seasons (Fig. 2B). Tarsus length was negatively related to RTL in nestlings, unrelated to RTL in fledglings, and positively related to RTL in subadults (Fig. 2C). Males had slightly longer telomeres than females, prarticularly at the fledgling and subadult stages (Fig. 2D), although this effect was weak.

##### Early-life telomere shortening

Longitudinal data showed that early-life RTL was positively, but weakly, related to adult RTL (R2 = 0.022; t = 2.40; P = 0.017; Fig. 3A). RTL was significantly lower than zero in nestlings (t = -4.91; P < 0.001), but not in fledglings (t = 0.07; P = 0.94) or subadults (t = 0.81; P = 0.42). In line with this, RTL increased with age in early life (R2 = 0.04; t = 3.51; P < 0.001), suggesting that telomere shortening was highest shortly after the nestling phase. In nestlings, RTL did not vary with the time between sampling events (R2 0.003; t = 0.47; P = 0.636). However, for fledglings and subadults RTL decreased with the length of time between sampling events (R2 = 0.02; t = -2.20; P = 0.029). In flesdglings and subadults, RTL did not decrease below zero until ~3 years between sampling events was allowed, and confidence limits overlapped zero regardless of the amount of time between sampling events (Fig 3B). Together, these results suggest that the majority of observable telomere shortening in Seychelles warblers occurs in the first six months of life. We therefore restricted the remaining analyses of RTL to birds sampled as nestlings.

RTL did not differ significantly among cohorts (F = 0.76; P 0.699), although power for this analysis was limited as longitudinal sample sizes within cohorts was low (Fig. 3C). Model selection revealed that the top model explaining RTL was the null model (Table S2), and confidence limits for all explanatory terms overlapped zero (Fig. 4A).

##### Early-life telomere dynamics and survival

Early-life RTL was not related to survival to adulthood (estimate = -0.58; P = 0.08), with no interaction effect between RTL and juvenile age on survival to adulthood (estimate = 0.07; P = 0.26). To test whether cohort-level effects on RTL influenced survival we calculated cohort-level RTL (i.e. the median RTL for each cohort) and added this term to a GLMM, and found that cofort-level RTL was not related to survival to adulthood (estimate = -1.04; P = 0.14), with no interaction with juvenile age (estimate = 0.32; P = 0.16). Using the longitudinal data, we found that RTL had no effect on survival to the year after the second sampling event (estimate = -0.77; P = 0.75).

Cox regression showed no effect of individual-level or cohort-level RTL on survival later in life (individual-level: estimate = 0.15, P = 0.28; cohort-level: estimate = 1.25, P = 0.07), and no interaction between RTL and age on survival (individual-level: estimate = -0.05, P = 0.23; cohort-level: estimate = 0.05, P = 0.80). RTL was not related to longer-term survival (estimate 0.515, P = 0.223).

### Discussion

Here we use one of the largest datasets to date to assess the relationships between early-life conditions, telomere length and survival in a closed population of Seychelles warblers. We found that, as in other studies, telomeres are lost at the greatest rate very early in life. We found that telomere dynamics are subject to strong seasonal and cohort effects, but that temporal variation in RTL is not related to any of the environmental variables tested. Within seasons, we found no evidence that the ecological or social environment influenced telomere dynamics, and we found no evidence that early-life telomere length is related to juvenile or later-life survival.

Our study adds the the growing number showing that telomere length decreases most rapidly in early life (e.g. Frenck et al. 1998; Haussmann et al. 2003; Heidinger et al. 2012). In the Seychelles warbler, although cross-sectional and longitudinal data show that RTL at the population level decreases with age throughout the warbler lifespan (Fig. 1), we found that individual-level telomere shortening can only be detected very early in life (Fig. 3).

Model averaging showed that in addition to juvenile age, tarsus length was related to juvenile RTL in an age-dependent manner (Fig. 2). However, this most likely an additional age effect - in passerine birds tarsus length is correlated with age during the nestling stage, but not after fledging (Ricklefs 1976). Telomere loss is most rapid early in life due to ongoing cell replication (Frenck et al. 1998), and a negative correlation between RTL and body size in early life is therefore expected.

Perhaps the clearest result from our study is that RTL varies among cohorts. Very few studies have shown that temporal variation in telomere dynamics occurs in natural populations, these have been limited to just two seasons (Mizutani et al. 2013; Watson et al. 2015). The long-term Seychelles warbler dataset has allowed us to show that temporal variation in telomere dynamics does occur at the population level over longer time periods. We found that this temporal variation was related to population density, and the season. The difference in RTL between birds born in summer and winter seasons is more surprising, especially given the strength and directionality of the effect. We find that birds born in winter seasons having longer telomeres, despite the . One possible explanation for this is that parents in good condition are more likely to breed

We suspect that the novelty in our finding temporal variation in telomere dynamics within a population is more due to a lack of available long-term datasets with telomere screening, rather than the Seychelles warbler being unique. Indeed, the population on Cousin is relatively stable in comparison with other populations, which can undergo large fluctuations in size (e.g. Coulson et al. 2001). If our findings are replicated in other systems and population-level variation in early-life telomere dynamics is common in nature, this has potential consequences for our understanding of telomere dynamics in natural populations. In particular, our findings suggest that the telomere dynamics of a population at a given point in time represent a snapshot of a temporally varying process. More research is now needed within and across multiple cohorts and populations to better understand how how and why population-level telomere dynamics vary over space and time.

Within cohorts, we found no evidence that any of the social or environmental variables we measured affect juvenile RTL. This is surprising, as a poor social and ecological environment is known to be detrimental to juvenile Seychelles warblers, both in terms of oxidative stress and later life survival. Possible explanations for this finding include i) temporal variation in RTL in our data imasks our ability to detect spatial trends, ii) within-cohort variation in RTL is explained by a variable that we have not included in our analyses, or iii) that effects are generally weak and levels of noise in our telomere measurements and/or ecological data preclude detection of significant effects. One thing unclear at present is to what extent early-life telomere length reflects inheritance (Asghar et al. 2014; e.g. Becker et al. 2015), and future research on the Seychelles warbler will examine, in a quantitative genetic framework, how genetic and environmental components, and their interactions, affect telomere dynamics and senescence in natural populations.

While the relationship between mortality and telomeres in adults has been established for some time (Cawthon et al. 2003), only recently has the link between later-life survival and early-life telomere dynamics been studied. In captive zebra finches, juvenile telomere length predicts late-life survival (Heidinger et al. 2012), and in wild bird populations survival to the nestling phase (Watson et al. 2015), and survival to adulthood (Boonekamp et al. 2014), have been linked with early-life telomere dynamics.

There are numerous avenues for future research into telomere evolutionary ecology in this system and others. Here we have considered survival, but telomere length and shortening in early life may also be linked to other components of fitness (reviewed in Monaghan 2014). Reproductive senescence occurs in the Seychelles warbler (reviewed in Hammers et al. 2015), making this system well suited to examining how telomere length predicts lifetime reproductive success. We expect that by gaining a fuller understanding of telomere dynamics in natural populations, the fields of life-history evolution and evolutionary ecology will be greatly enhanced.

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