**Spatiotemporal variation in lifelong telomere dynamics in the Seychelles warbler**

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**Running head:** Early-life telomeres

### Abstract

1. Understanding the costs individuals and populations face throughout their lifetimes is fundamental to understanding life-history evolution and population dynamics. Telomeres, the protective caps at the ends of chromosomes, shorten in response to oxidative stress, and telomere shortening is correlated with reduced survival. Thus, telomere dynamics may help us quantify individual variation in costs, and enhance our understanding individual variation in natural populations.
2. The Seychelles warbler (*Acrocephalus sechellensis*) is an excellent model system for disentangling the causes and consequences of individual variation in telomere dynamics. Here we study spatiotemporal variation in lifelong telomere dynamics in the Seychelles warbler. We combine long-term ecological data with one the largest longitudinal telomere datasets to date, consisting of 1828 samples from multiple cohorts spanning 19 years. We provide a detailed analysis of how telomere dynamics vary over individual lifespans, and with spatiotemporal variation in the social and ecological environment.
3. We found that telomere length decreases with cross-sectional and longitudinal measures of age, and that telomere length decreases most rapidly early in life. However, both cross-sectional and longitudinal data suggested that telomere length increases occurs in some individuals for short periods, and using a large number of repeated measurements we show statistically that this increase cannot be explained solely by qPCR measurement error.
4. Telomere length and rates of shortening varied markedly both within and among cohorts, and cohort-level differences in telomere length persisted throughout individual lifespan. Temporal variation in telomere length was positively associated with island-wide temporal variation in food availability. The other significant predictors of telomere length were sex, with males having longer telomeres than females, and tarus length, which affected telomere length in a sex-specific manner.
5. Our comprehensive, long-term data show that in natural populations, telomere dynamics vary enormously over both space and time. Ascertaining what explains this variation requires combining large sample sizes spanning multiple cohorts, and long-term ecological data.

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

**Data archival location:** This manuscript was written in R Markdown (<http://rmarkdown.rstudio.com/>). All data and scripts required to reproduce the manuscript, figures and analyses will be made available on GitHub.

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### Introduction

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 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 in this species (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 over the entire lifespans across multiple cohorts of the Seychelles warbler.

### 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' main breeding season runs from June-August, 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 where territory quality estimates were not available for a specific year we used the average value for that territory across years (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 relative telomere length (RTL) for all samples using a quantitative PCR (qPCR) assay of telomeres and a GAPDH control gene, using the molecular methods outlined by Bebbington *et al.* (**???**). We assessed repeatability of RTL using the rptR package in R (R Development Core Team 2011).

For a subset of birds we had longitudinal data, with an additional samples taken at different ages. For these individuals we calculated the within-individual change in RTL by subtracting RTL at time point *t* from RTL at timepoint *t* + 1 (hereafter RTL). Negative values of RTL reflect telomere shortening, while positive values reflect telomere lengthening.

##### Statistical analyses

We performed all statistical analyses using R. We first tested the relationship between RTL and age using linear mixed models (LMMs) with individual ID as a random effect, carried out in the lme4 package (**???**). To test whether the decrease in telomere length with age is greatest in early life, we compared LMMs with age (in months) and log-transformed age, using a likelihood ratio test. We also tested whether telomores shorten with age in individuals with samples from multiple timepoints, using a LMM of RTL and age (a longitudinal measure based on within-subject centring; Pol and Wright 2009). To test how telomere length, and its relationship with age, varied among cohorts, we compared the above LMMs with models that included birth year and catch year as additional random effects. We also compared a model in which only the intercept (i.e. RTL) was allowed to vary among among cohorts or years, to one in which the slope of the relationship between RTL and age was also allowed to vary.

Because we observed apparent telomere lengthening in our data, we used the entire Seychelles warbler dataset to test whether this lengthening could be explained by measurement error. Using all birds (adults and juveniles) with at least two telomere measurement, we calculated RTL for each pair of samples (for example, for a bird with three longitudinal measurements throughout their life we calculated the change in RTL between the first and second sample and between the second and third sample). We then compared these longitudinal changes in telomere length to sampling error, using all samples where we had at least two repeat telomere measurements for the same sample (i.e. completely seperate reactions run on separate plates: N = 422 measurements from 293 samples). We calculated RTL between pairs of repeat measurements within samples in exactly the same way as for across samples, except that repeat measurements were ordered by the date at the qPCR was run rather than by age. We first compared the variance in RTL within and among individuals using a Levene's test. Then, to separately test whether the extent of telomere shortening and lenghtening within individuals was greater than expected by measurement error, we split RTL measurements into groups in which shortening (RTL < 0) and lengthening RTL (RTL > 0) occurred, and tested whether RTL among samples was significantly different between within-sample and across-sample measurements, using Wilcoxon tests.

We used LMMs to test how variation in early-life environmental and social conditions influenced telomere length within cohorts. We created a full model with RTL as a response variable, alongside the following explanatory variables: age, tarsus length, body mass, sex, annual food availability, territory quality, population density (an annual measure estimated from the summer breeding census) and the number of birds and the number of helping subordinate birds present in the territory. As random effects we included catch year and individual identity. We report model estimates and confidence intervals for all effects included in the full model. To obtain an estimate of explanatory power, we calculated marginal R^2 (just incoporatng fixed effects) and conditional R^2 (incorporating fixed and random effects) following Nakagawa & Schielzeth (**???**) and Johnson (**???**), respectively As a complementary approach, we also performed model averaging, using the MuMIn package in R (Bartoń 2012). Model selection was performed using the full model described above 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* = 661) we repeated the above analyses of telomere dynamics, replacing telomere length with RTL as the response variable, and excluding the plate ID random effect (as each measurement of RTL was based on two or more measurements, and so run on multiple plates).

### Results

We measured telomere lengths using a total of 1828 unique samples from juvenile and adult Seychelles warblers. Efficiencies (mean s.d.) for our telomere and GAPDH reactions were (1.78 0.05) and (1.92 0.04) respectively. Inter-plate repeatability of RTL, based on 422 samples measured at least twice, was 0.68 (CI = 0.64-0.71)

##### Telomere dynamics and age

Using cross-sectional data we found that RTL decreased with age in the Seychelles warbler (LMM: estimate = -0.007, CIs = -0.010, -0.004). RTL also decreased with log-transformed age (LMM: estimate = -0.05, CIs = -0.06, -0.04), and this model was a significantly better fit than one modelling a linear relationship between RTL and age (P = < 0.001), suggesting that telomere length decreased at the greatest rate in early life - something that was evident from the raw data (Fig. 1A). Also worth noting is that while the overall trend was for decreasing RTL, there were appoarent increases in cross-sectional telomere length after one year of age and at several points later in life (Fig. 1A). Using longitudinal data, we found that RTL decreased with Age (LMM: estimate = -0.013, CIs = -0.021, -0.006), showing that telomere length decreases with age within individuals (Fig. 1B).

Including birth year as a random effect in a LMM of RTL and (log) age resulted in a significantly improved model (conditional R2 = 0.24) to one with only individual ID and plate ID as random effects (conditional R2 = 0.24; likelihood ratio test, P < 0.001). Allowing the slope of the age effect to vary among birth years resulted in a slightly significant improvement on one in which only the intercept was allowed to vary (conditional R2 = 0.28; P = 0.008; Fig. 2B). However, a model including catch year and individual ID as random effects explaine dmore variation in RTL than one including birth year (conditional R2 = 0.21; P < 0.001; Fig 2C) suggesting that variation among catch years was greater than among cohorts (Fig. 2C), and a model that allowed the slope of the age effect to vary among catch years was better still (conditional R2 = 0.23; P = < 0.001; Fig. 2D). Temporal dynamics of longitudinal telomere data were similar to the cross-sectional data. A model of RTL and Age including catch year as a random effect was better than one that did not allow for temporal variation in RTL (P = 0.086), and better than one with birth year as a random effect (P < 0.001). However a model allowing the slope of the Age effect was not better than one in which only the intercept varied (P = 0.936).

Within individuals, longitudinal data showed that RTL measurements taken from different timepoints were weakly, but significantly, positvely correlated (R2 = 0.006; t = 1.94; P = 0.053; Fig. 3A). Although both or cross-sectional and longitudinal data indicated a general trend of telomere shortening with age, we found that 44% of our RTL measurments were positive, and thus indicative of telomere lengthening. To test whether the apparent telomere lengthening in our dataset could be explained by measurement error, we compared variance in telomere length among repeat measurements of samples to the variance observed among different samples of the same individual (see methods for details). We found significantly higher variance in telomere length over individual lifetimes compared to among sample replicates (Levene's test: F = 44.52; P < 0.001; Fig. 3B). Importantly, splitting the longitudinal data into instances of shortening (RTL < 0) and lenghtning (RTL > 0) revealed that not only did we observe significantly more shortening within individuals compared to within samples (Wilcoxon test: W = 3.476110^{4}; P < 0.001), but also significantly more lengthening (W = 31336; P < 0.001; Fig. 3B).

##### Spatiotemporal variation in telomere dynamics and the environment

A LMM revealed that age, tarsus length, sex and annual variation in food availability were significantly related to RTL (Table 1). Tarsus length was negatively related to RTL and males had longer telomeres than females (Table 1; Fig. 4A), while insect availability was positively related to RTL (Table 1; Fig. 4B). The full model was weak in terms of explanatory power of fixed effects (marginal R2 = 0.06), although including the random effect terms increased this substantially (conditional R2 = 0.23) The model averaging approach yielded qualitatively identical results to the full LMM, with the same explanatory variables 'significant' in terms of being retained in top models, and having model-averaged confidence intervals not overlapping zero (Table S1; Fig. S1). One interesting finding from the model selection was that sex only appeared in top models where tarsus length was also present (Table S1). In accordance with this, when tarsus length was removed from the full model sex was no longer significant (estimate = 0.009, CIs = -0.013, 0.031), and a sex x tarsus interaction was significant when included (estimate = 0.023, CIs = 0.004, 0.041); RTL increased with tarsus length in males, but decreased in females (Fig. 4A). No social or ecological environmental variables were significant predictors of RTL, regardless of whether we interpreted the full model (Table 2), or used model averaging (Table S2; Fig. S2).

### Discussion

Here we use a long-term, multi-cohort dataset to assess the relationships between the spatiotemporal variation in the ecological environment and lifelong telomere dynamics in a closed population of Seychelles warblers. We found that telomeres shorten over time, and that telomere loss is greatest in early life. However, bouts of apparent telomere lengthening occur, and the extent of this lengthening cannot be explained solely by measurement error. We then found that telomere dynamics vary among sexes, and over time, with this temporal variation is related to fluctuations in food availability.

Our study adds the the now substantial body of literature showing that telomere length decreases with age in both humans and wild animals, and that this decrease is most rapid in early life (**???**; e.g. Frenck et al. 1998; Haussmann et al. 2003; Heidinger et al. 2012). However, using longitudinal data we found that within-individual telomere measurments were only weakly correlated, and that consistent telomere shortening only occurs early in life, after which both shortening and lengthening occurs. This is in contrast to other avian studies where birds were reared in laboratory (Heidinger et al. 2012) or nest-box (Boonekamp et al. 2014) conditions, in which juvenile and adult telomere length were highly correlated, and individual-level telomere shortening clearly occurs throughout the juvenile period, and into adulthood. However, in accordance with our study, a recent study on a wild population of Soay sheep (*Ovis aries*) found only a very weak correlation between juvenile and adult RTL, and a highly complex pattern of telomere dynamics across the lifespan (**???**).

We also found evidence for sex-specific telomere dynamics in Seychelles warblers, with males having longer-telomeres than females, in and that this sex-difference interacts with tarsus length. In Seychelles warblers the effect of telomere length on survival is strongest for males (Barrett et al. 2013). Together, these results suggest that sex effects should be considered when studying any aspect of telomere dynamics (see also Barrett and Richardson 2011).

Perhaps the clearest result from our study is that RTL, among cohorts, and among sampling years. A few studies have now shown that temporal variation in telomere dynamics occurs in natural populations, although these have been limited in the number of 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 can occur over substantial time periods. Moreover, because of the large number of cohorts we have been able to show statistically that that temporal variation in RTL is related to variation in food availability (Fig. 4B). If cohort-level variation in telomere dynamics continues to be found in other systems, 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.

Despite substantial cohort-level variation in RTL, 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 (Van de Crommenacker et al. 2011; Brouwer et al. 2012). An explanation for this could be that temporal variation in RTL in our data masks our ability to detect spatial trends. However, this is unlikely as we controlled for cohort ID, and within-cohort variation in RTL greatly exceeds among-cohort variation (Fig. 1C). A second possibility is that effects are generally weak and levels of noise in our telomere measurements and/or ecological data preclude detection of significant effects. While noise in telomere measurements and problems with power are a problem in any study using qPCR to measure telomere length (Nussey et al. 2014), our sample sizes and levels of repeatability compare favourably with other studies of natural populations that have found significant effects of the early-life environment on telomere length (e.g. **???**). More likely is that early-life RTL in the Seychelles warbler is explained by a set of variables that we have not measured. A key question to be addressed is the extent to which early-life RTL reflects inheritance (Asghar et al. 2014; e.g. Becker et al. 2015). This is important, as inheritance may capture a large amount of unexplained variation in early-life RTL within our dataset. 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.

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. It is worth noting that 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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