# Population differences in the length, early-life dynamics, and heritability of telomeres among European pied flycatchers

Running title: Population differences in telomere length

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**Abstract**

Telomere length and shortening rate are increasingly used as biomarkers for long-term costs in ecological and evolutionary studies because of their relationships with survival and fitness. Telomere length can be heritable, but both early-life conditions and later-life stressors can create variation in telomere shortening rate. Studies on between-population telomere length and dynamics are mostly lacking, despite the expectation that populations exposed to varying environmental constraints would present divergent telomere length patterns. Pied flycatcher (*Ficedula hypoleuca*) is a passerine bird spending the non-breeding period in sub-Saharan Africa but breeding across Eurasia (from Spain to western Siberia). Populations show marked differences in migration distance, genetics, breeding parameters, and egg components. We studied the large-scale variation of telomere length, early-life dynamics and heritability in the pied flycatcher by comparing six European populations across a north-south gradient (Finland, Estonia, England and Spain). There were clear population differences in telomere length, with English birds having the longest telomeres, followed by Spanish and lastly by Estonian and Finnish birds. Early-life telomere shortening rate tended to vary between populations, and faster nestling growth affected telomeres more negatively in northern than southern populations. The heritability of telomere length was moderate ($h^2$ = 0.34 – 0.40), with stronger heritability to paternal than maternal telomere length. There was also evidence indicating that the level of paternal heritability could differ between populations. While the sources of between-population differences in telomere-related biology remain to be identified (*i.e.* genetics, environmental factors), our study illustrates the need to expand telomere studies at the between-population level.

Keywords: ageing, biogeography, bird, habitat, migration, telomere length

## Introduction

Telomeres, the capping structures of linear chromosomes, have a crucial role in maintaining genomic integrity and cell viability (Blackburn, 1991; Blackburn, Epel, & Lin, 2015). They shorten with cell divisions and the shortening can be accentuated by cellular and external stressors, such as oxidative stress or substantially high energy demands (Casagrande & Hau, 2019; Levy, Allsopp, Futcher, Greider, & Harley, 1992; Sophie Reichert & Stier, 2017). As short telomeres are associated with ageing phenotypes (Campisi, Kim, Lim, & Rubio, 2001), telomere length is increasingly used as a biomarker of ageing to predict survival and fitness (Monaghan, Eisenberg, Harrington, & Nussey, 2018). To date in wild populations, telomere length has been associated with past stress exposure (Chatelain, Drobniak, & Szulkin, 2020), individual quality (Angelier, Weimerskirch, Barbraud, & Chastel, 2019), fitness (Eastwood et al., 2019) and overall mortality (Wilbourn et al., 2018), suggesting usefulness of telomere length as a biomarker for long-term costs in wild animals.

Most telomere shortening happens during early life growth (Spurgin et al., 2018; Stier, Metcalfe, & Monaghan, 2020), and fast growth has been suggested to accelerate telomere shortening (Monaghan & Ozanne, 2018). Early-life conditions, with the associated hormone levels (Casagrande et al., 2020; Stier, Hsu, et al., 2020), competition (Cram, Monaghan, Gillespie, & Clutton-Brock, 2017; Young et al., 2017), and nutrition deficiency (Nettle et al., 2017) can affect individual telomere length trajectories and thus could promote individual differences in longevity. Later-life stressors, such as predation risk (Kärkkäinen et al., 2019), parasitic infections (Asghar et al., 2015), low prey abundance (Spurgin et al., 2018), reproductive effort (Bauch, Gatt, Granadeiro, Verhulst, & Catry, 2020; López-Arrabé et al., 2018; Sudyka, Arct, Drobniak, Gustafsson, & Cichoń, 2019) and migration (Bauer, Heidinger, Ketterson, & Greives, 2016) can create further between-individual differences in telomere length. Telomere length and dynamics have also been associated with genetic polymorphism (Eisenberg, 2019; Karell, Bensch, Ahola, & Asghar, 2017). Telomere length is somewhat heritable, but the heritability estimates in the wild are rather scarce and highly variable, ranging from null (0) to high levels (1) (Dugdale & Richardson, 2018), leaving unclear its potential evolutionary role.

While within-population telomere length patterns have been widely examined (*i.e.* all the examples cited above), studies on among-population telomere length and dynamics are still mostly lacking. Species distributed over vast latitudinal gradients face different and variable environmental conditions, for example temperature and seasonality (Willig, Kaufman, & Stevens, 2003), which might ultimately influence telomere dynamics (Angelier, Costantini, Blévin, & Chastel, 2018). Accordingly, telomere length has been shown to decrease at higher latitudes in American black bears (*Ursus americanus*) (Kirby, Alldredge, & Pauli, 2017). Similar to latitudinal gradients, environmental factors can change with increasing elevation (Willig et al., 2003), for example, nestlings from two tit species (*Parus* spp.) showed faster telomere shortening at

higher altitudes (Stier et al., 2016). Therefore, populations or subpopulations of species facing specific energetic demands and environmental stressors may display divergent patterns of telomere length and dynamics, and ultimately ageing rates (*e.g.* Ibáñez-Álamo et al., 2018). Knowledge of the mechanisms driving individuals' telomere length trajectories across populations could help in understanding life history evolution in different environments and the resilience of populations to environmental change, as short telomeres could also be indicative of local extinction risk (Dupoué et al., 2017). In addition, potential differences in telomere length within species but between populations question the use of 'species' data in meta-analyses and comparative studies when there is data from only one population.

Our study species, the pied flycatcher (*Ficedula hypoleuca*), is a small, insectivorous migratory passerine that breeds over a large area of Eurasia from Spain to western Siberia in a wide range of forest habitats, from high altitude forests to temperate deciduous and boreal coniferous forests (Lundberg & Alatalo, 1992). In the autumn, pied flycatchers from across the breeding range migrate through Iberian peninsula to spend the non-breeding period in sub-Saharan Africa (Chernetsov, Kishkinev, Gashkov, Kosarev, & Bolshakov, 2008; Lundberg & Alatalo, 1992; Ouwehand et al., 2016). Consequently, different pied flycatcher populations experience marked differences in the distance (and hence duration) of their migration. Migratory flight can increase metabolic rate (Kvist & Lindström, 2001), which in turn might accelerate telomere shortening either through increased oxidative stress (Reichert & Stier, 2017) or through metabolic adjustments (Casagrande & Hau, 2019). Thus, pied flycatchers with a longer migration distances might exhibit shorter telomeres than those with a shorter migration. There is evidence that pied flycatcher females breeding in Spain, in the southern part of the breeding range and with the shortest migration, show higher adult survival, natal recruitment rate and delayed onset of reproductive ageing compared to pied flycatchers breeding further north (Sanz & Moreno, 2000). Furthermore, pied flycatcher populations across the breeding range are genetically differentiated from each other to some extent, with birds breeding in England, and in mountainous habitats in Spain and central Europe showing the most differentiation (Haavie, Sætre, & Moum, 2000; Lehtonen et al., 2012; Lehtonen et al., 2009), which could affect among-population telomere length and dynamics. European pied flycatcher populations also differ in breeding initiation, clutch size, number of fledglings (Sanz, 1997), and various egg characteristics (Morales et al., 2013; Ruuskanen et al., 2011), which might ultimately influence population-specific telomere length trajectories.

Here, we studied large-scale variation in the telomere biology of pied flycatchers by sampling chicks and adult birds from six breeding populations across a north-south gradient across Europe. We specifically examined 1) overall patterns of telomere length across populations and life stages, 2) associations between telomere length and migration distance, 3) early-life telomere dynamics and body mass growth across populations, 4) relationships between telomere shortening rate and body mass growth rate and 5) heritability of telomere length using parent-offspring regressions.

## Methods

*Study populations*

Data for this study were collected during the 2019 breeding season from six different pied flycatcher populations along the south-north axis of the breeding range: Valsaín, central Spain (40°54'N, 4°01'W), La Hiruela, central Spain (41°04'N, 3°27'W), East Dartmoor, southern England (50°36'N, 3°43'W), Kilingi-Nõmme, southern Estonia (58°7'N, 25°5'E), Turku, southern Finland (60°25'N, 22°10'E), and Oulu, northern Finland (65°0'N, 25°48'E). All birds were breeding in nest boxes in study areas established several years or more before this study.

*Sample collection*

Between early May and early June pied flycatcher nests were monitored in each study area for laying date (pied flycatchers lay one egg per day), clutch size (typically 5-8 eggs) and hatching date (on average 14 days from the start of incubation). The nestling period from hatching to fledging is *ca.* 15-17 days. One random chick per nest was sampled at days 5 and 12 (hatching day = day 0). By day 12, most of the chicks' structural growth is already complete and the mass gain has peaked and flattened (Lundberg & Alatalo, 1992), while sampling later than this may cause pre-mature fledging. Additionally, the parent birds were caught and sampled when their chicks were around 10 days old. Approximately 60 birds per population (20 chicks, 20 females, and 20 males; see exact sample sizes in figure legends) were sampled. All birds were weighed after blood sampling. In case the exact age of an adult could not be determined based on ringing information, the adults were aged based on feather characteristics (Svensson, 1992). All adults were categorized either as a one-year-old (young) or older (old).

The same blood sampling protocol including blood storage buffers was applied to all populations to eliminate differences in sample collection and storage, as this might affect the subsequent telomere measurements (Reichert et al., 2017). Blood samples (10-30 µl from adults and 12-day chicks, 10 µl from 5-day chicks) were collected by puncturing the brachial vein with a sterile needle and collecting the blood with a non-heparinized capillary tube. Blood was diluted with *ca.* 65 µl of PBS for storage. The samples were kept cold while in the field and stored at -20° C at the end of the day. All the blood samples were shipped to University of Turku on dry ice for DNA extraction and telomere length quantification.

*Laboratory analyses*

All the laboratory work was conducted at the University of Turku by TK. Four months after sample collection, DNA was extracted from whole blood using a salt extraction alcohol precipitation method (Aljanabi & Martinez, 1997). Extracted DNA was diluted with BE buffer (Macherey-Nagel, Düren, Germany). DNA concentration and purity were quantified using ND-1000-Spectrophotometer (NanoDrop Technologies,

145 Wilmington, USA). DNA integrity was checked using gel electrophoresis (50 ng DNA, 0.8% agarose gel at 100
146 mV for 60 min using MidoriGreen staining) on 25 randomly selected samples and was deemed satisfactory.
147 Diluted DNA was aliquoted and stored in -20° C until telomere length assessment.

148 Real-time quantitative PCR (qPCR) was used to assess relative telomere length, as previously
149 described in birds (Criscuolo et al., 2009) and validated in the pied flycatcher (Kärkkäinen et al., 2019). qPCR
150 quantifies the amount of telomeric sequence (T) relative to the amount of single copy gene sequence (SCG)
151 resulting in relative telomere length (T/S ratio). Here, we used RAG1 as a SCG (verified as single copy using a
152 BLAST analysis on the collared flycatcher *Ficedula albicollis* genome), as previously used in Kärkkäinen et al.
153 (2020). Forward and reverse RAG1 primers were 5'-GCAGATGAACTGGAGGCTATAA-3' and 5'-
154 CAGCTGAGAAACGTGTTGATTC-3' respectively, and forward and reverse telomere primers were 5'-
155 CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' (Tel-1b) and 5'-
156 GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3' (Tel-2b). Both primers were used at a final
157 concentration of 200nM. For the qPCR assay, 5ng of DNA per reaction was used in a total volume of 10µl (8µl
158 of master mix+2µl of DNA). The master mix contained 0.1µl of each primer, 2.8µl of water and 5µl of
159 SensiFAST SYBR Lo-ROX master mix (Bioline, London, UK) per reaction.

160 Due to the closing down of many laboratory service providers in 2020 following the worldwide
161 Covid-19 pandemic, the qPCR analyses were performed on two instruments. First, 71% of the samples were
162 analyzed with QuantStudio™ 12 K Flex Real-Time PCR System (Thermo Fisher) using 384-well qPCR plates,
163 while the rest of the samples were analysed with MicPCR (Magnetic Induction Cycler PCR Machine, Bio
164 Molecular Systems) fitting 48-well plates. A subset of samples (n = 21) initially analysed with QuantStudio
165 were rerun with MicPCR, and the technical repeatability between the two measurements was 0.768 (95% CI
166 [0.54, 0.893], P<0.001).

167 In QuantStudio the telomere and RAG1 reactions were run in triplicates adjacent to each other
168 on the same plate. Each plate contained one golden sample that was run twice, one internal standard, and
169 one negative control. The qPCR conditions were: an initial denaturation (1 cycle of 3min at 95°C), 40 cycles
170 with first step of 10s at 95°C, second step of 15s at 58°C and third step of 10s at 72°C, with melting curve
171 analysis in the end. In the MicPCR, the samples were run in duplicates and the telomere and RAG1 reactions
172 were performed on separate plates. Each plate contained the golden sample twice, and the internal standard.
173 The qPCR conditions in the MicPCR were: an initial denaturation (1 cycle of 3min at 95°C), 25/40 cycles
174 (telomere/RAG1) with first step of 5s at 95°C, and second step of 25s at 60°C, with melting curve analysis in
175 the end. Samples from the same individual and from the same nest were analyzed on the same plate, and
176 samples from different origins were evenly distributed between all the plates.

LinRegPCR (Ruijter et al., 2009) was used to determine the baseline fluorescence, the qPCR efficiencies and the quantification cycle (Cq) values. Relative telomere length (T/S ratio, hereafter telomere length) was calculated based on plate-specific efficiencies (mean ± s.d. efficiencies were 1.90 ± 0.02 for RAG1 and 1.89 ± 0.06 for telomere) using the mathematical model presented in Pfaffl et al. (2001). Technical repeatability based on triplicate measurements of telomere length was 0.957 (95% CI [0.951, 0.962], $p<0.001$), and inter-plate repeatability based on samples measured on more than one plate was 0.897 (95% CI [0.831, 0.932], $p<0.001$). Age-adjusted within-individual repeatability of telomere length in chicks was 0.373 (95% CI [0.217, 0.518], $p<0.001$), which is close to the average value found for qPCR studies (Kärkkäinen, Briga, Laaksonen, & Stier, 2021).

*Statistical methods*

We used linear models, linear mixed models, and correlation analyses to study population differences in telomere length, chick growth, and telomere length heritability. Statistical analyses were conducted with SAS statistical software version 9.4 (SAS Institute, Cary, NC, USA). The models were estimated using restricted maximum likelihood (REML) and Kenward–Roger method was used to calculate degrees of freedom of fixed factors, and to assess parameter estimates and their standard errors. Normality and heteroscedasticity assumptions were checked visually by plotting the models' residuals (normal probability plot, histogram, boxplot, and linear predictor plot – results not shown).

We started by examining potential population differences in telomere length by fitting a model with telomere length as the dependent variable, age (5-day chick, 12-day chick, one-year-old adult, older adult), population and their interaction as explanatory factors, and nest ID, bird ID, and qPCR plate as random effects. However, as there was no significant difference in telomere length between the one-year-old and older adults (post hoc pairwise comparison $p = 0.64$), the adults were grouped together and the same model was run with life stage (5-day chick, 12-day chick, adult), population and their interaction as independent variables. As the interaction term was not significant (see Results), it was removed from the final model that included the main effects of life stage and population. To further examine whether the potential population differences in telomere length were related to migration distance, we separately tested for the correlations between migration distance and adult telomere length (average TL of a breeding pair), and migration distance and chick TL at day 12. Since all the individuals in a population have the same distance value, only the average TL values per population were included in these analyses. The Spanish populations breed so close to each other compared to other populations in Europe (distance in straight line: La Hiruela – Valsaín: 53 km) that they were considered as one population in this analysis. Migration distance was calculated as a straight distance in km between breeding site and non-breeding site coordinates using latitude/longitude distance calculator (www.nhc.noaa.gov/gccalc.shtml). Non-breeding site coordinates were estimated for populations

210 from Finland and England according to data in Ouwehand et al. (2016) and Bell et al. (in review). Estonian
211 birds were assumed to winter in the same areas as Finnish birds, and Spanish populations in the same areas
212 as English birds.

213 To examine patterns of chick growth and telomere dynamics between populations in more
214 detail, we first fitted three models with chick body mass at day 5, body mass at day 12, and growth rate (body
215 mass change between day 5 and day 12) as dependent variables and population as fixed effect in all the
216 models. Additionally, the body mass change model included the initial body mass (day 5) as a covariate. Then,
217 we fitted a model with chick telomere change value (change between day 5 and day 12) as the dependent
218 variable, population as an explanatory variable, and qPCR plate as a random effect. Since the change in chick
219 telomere length was calculated by subtracting day 5 measurement from day 12 measurement (thus negative
220 values indicate telomere loss), regression to the mean was corrected by following the equations in Verhulst
221 et al. (2013). As growth may influence telomere dynamics, we ran six different models examining the effects
222 of body mass on telomeres between populations. Each dependent telomere variable (day 5, day 12, and
223 change) was tested with population and corresponding body mass as explanatory variables, first as main
224 effects and then also including the interaction term. qPCR plate was included as random effect in all the
225 models.

226 We used parent-offspring regressions to study heritability of telomere length. We studied the
227 effects of parental (mid-parent [*i.e.* average of paternal and maternal telomere length], paternal, and
228 maternal) telomere length while controlling for population, as well as the interactive effects of mid-
229 parent/paternal/maternal telomere length and population on chick telomere length at day 5 and day 12.
230 qPCR plate was included as a random effect in all models. When calculating heritabilities of traits that vary
231 with environment and age, like telomere length, parent-offspring regressions might either inflate (due to
232 common environmental effects) or underestimate (using different-aged individuals but not taking age-
233 related variation into account) the true heritability (Dugdale & Richardson, 2018). However, previous studies
234 have reported similar heritability estimates using parent-offspring regressions and 'animal models'
235 methodology that can better separate environmental effects from the genetic effects (Åkesson, Bensch,
236 Hasselquist, Tarka, & Hansson, 2008; Bauch, Boonekamp, Korsten, Mulder, & Verhulst, 2020), suggesting that
237 regression models can work as a tool to estimate heritability in the broader sense.

238

239 **Results**

240 While there were clear effects of both life stage and population on pied flycatcher relative
241 telomere length (Life stage: $F_{2, 250.4} = 56.05$, $p < .0001$; Population: $F_{5, 152.6} = 16.45$, $p < .0001$; Fig. 1), the general
242 pattern of telomere dynamics with age did not differ significantly between populations (Life stage ×

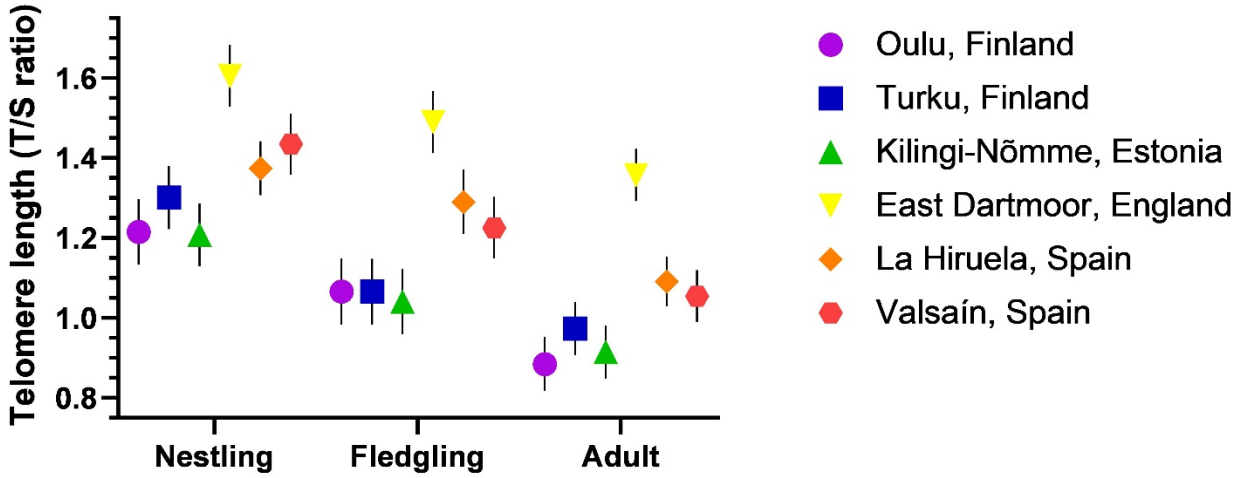


**Figure 1.** Relative telomere length in six pied flycatcher populations across a north-south gradient in Europe, from the early nestling period (Nestling; 5 days after hatching), to fledging (Fledgling; 12 days after hatching) and adulthood (Adult; end of the rearing period). Values are estimated marginal means ± s.e.m. Sample sizes [for Population: Nestling/Fledgling/Adult] are: Oulu: 19/19/41; Turku: 21/19/41; Kilingi-Nõmme: 22/20/43; East Dartmoor: 23/22/45; La Hiruela: 35/19/52; Valsaín: 24/23/49.

Population: $F_{10, 314.6} = 0.39$, $p = 0.95$; Fig. 1). Post hoc pairwise comparisons adjusted with the Tukey-Kramer method revealed that telomeres gradually shortened from day 5 to adulthood (Fig. 1, all p <.0001). Additionally, pied flycatchers from England (East Dartmoor) had significantly longer telomeres than any other population at all life stages (all p <.0002) and both Spanish populations (Valsaín and La Hiruela) had significantly longer telomeres than populations in northern Finland (Oulu) and Estonia (Kilingi-Nõmme) (Fig. 1; all p <0.032). There was no clear correlation between migration distance and relative telomere length, neither in adults (r= -0.57, p = 0.32, N = 5 populations) nor in fledglings (r = -0.69, p = 0.20, N = 5 populations) (Fig. S1).

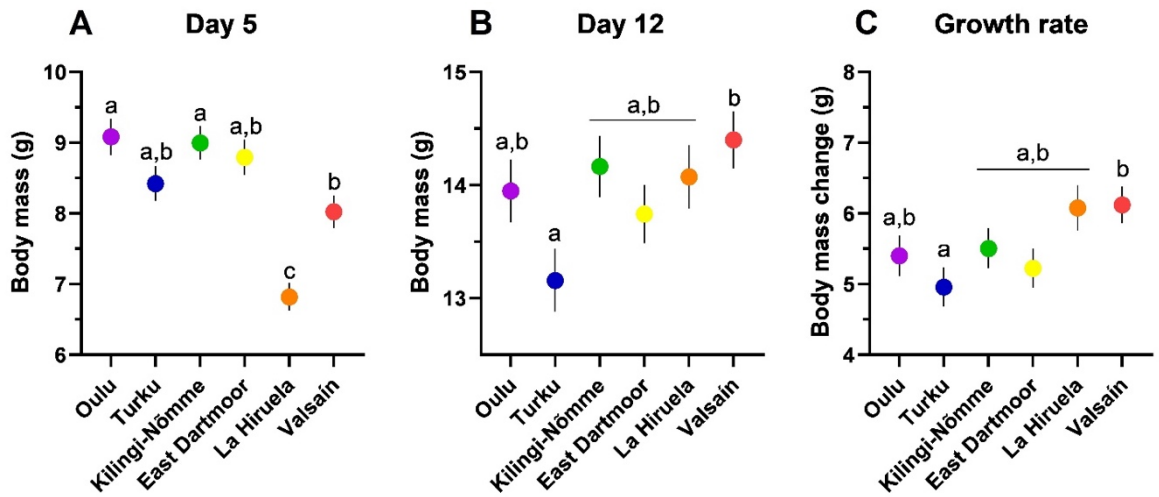


**Figure 2.** Pied flycatcher chick body mass at day 5 (A), day 12 (B) and growth rate (Δ mass between days 12 and 5; C) in six populations across a north-south gradient in Europe. Statistically significant differences after Tukey-Kramer adjustment for multiple comparisons are indicated with different letters. Values are estimated marginal means ± s.e.m. Sample sizes [for Population: Day5/Day12/Growth] are: Oulu, Finland: 19/19/17; Turku, Finland: 21/19/18; Kilingi-Nõmme, Estonia: 22/20/19; East Dartmoor, England: 20/22/18; La Hiruela, Spain: 33/19/18; Valsaín, Spain: 24/23/21.

While focusing specifically on the early-life (nestling) period, the population of origin had a strong effect on body mass at day 5 ($F_{5, 133}$ = 15.94, $p$ <.0001; Fig. 2A), day 12 ($F_{5, 116}$ = 2.53, $p$ = 0.03; Fig. 2B) and growth rate ($F_{5, 104}$ = 2.59, $p$ = 0.03; Fig. 2C). Overall, chicks from both Spanish populations were smaller at day 5 but gained more body mass between day 5 and 12 to reach a fledging body mass similar to the other populations (Fig. 2). At day 12 the only significant difference in body mass was between the lightest (Turku) and the heaviest (Valsaín) chicks. There was also an effect of the population of origin on telomere shortening rate, albeit non-significant ($F_{5, 86.38}$ = 2.14, $p$ = 0.068; Fig. 3). Chicks from England (East Dartmoor), Spain (only Valsaín population) and southern Finland (Turku) tended to have higher shortening rates than the three other populations, although no post hoc tests were conducted due to the non-significance of the main effect (Fig. S2).

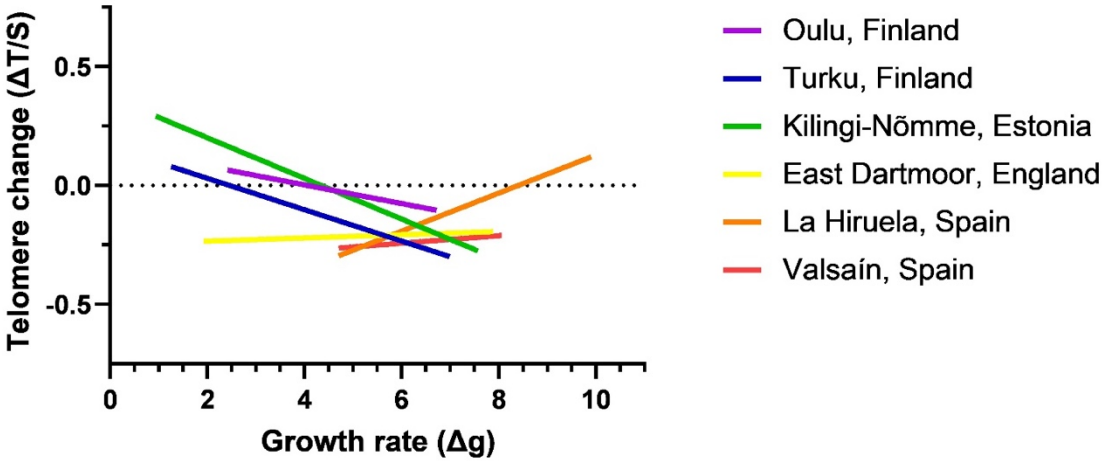


**Figure 3.** Association between growth rate (Δ mass between days 12 and 5) and telomere change (Δ telomere length between days 12 and 5) in pied flycatcher chicks in six populations across a north-south gradient in Europe. The interaction between population and growth rate was significant ($p$ = 0.04) in explaining variation in telomere change (see results for details). Values are fitted with simple linear regression lines and individual data points are not shown for clarity. Sample sizes [for Population] are: Oulu: 17; Turku: 18; Kilingi-Nõmme: 19; East Dartmoor: 18; La Hiruela: 18; Valsaín: 21.

Interestingly, while controlling for the population effect, chicks that were heavier at day 5 had shorter telomeres (β = -0.052 ± 0.02, $F_{1, 124.2}$ = 4.98, $p$ = 0.028) while there was no interaction between the population of origin and mass at day 5 in explaining telomere length at this age ($F_{5, 120.1}$ = 0.77, $p$ = 0.58). Such a relationship was not clear by day 12, although the direction of the relationship remained (β = -0.032 ± 0.03, $F_{1, 114.6}$ = 1.44, $p$ = 0.23). Also, there was a significant interaction between population of origin and growth rate in explaining variation in telomere shortening rate (Population × Growth rate: $F_{5, 88.49}$ = 2.40, $p$ = 0.04). Specifically, results suggest that fast growth was associated with faster telomere shortening in Finnish and Estonian populations, with the opposite relationship for English and Spanish populations (Fig. 3).

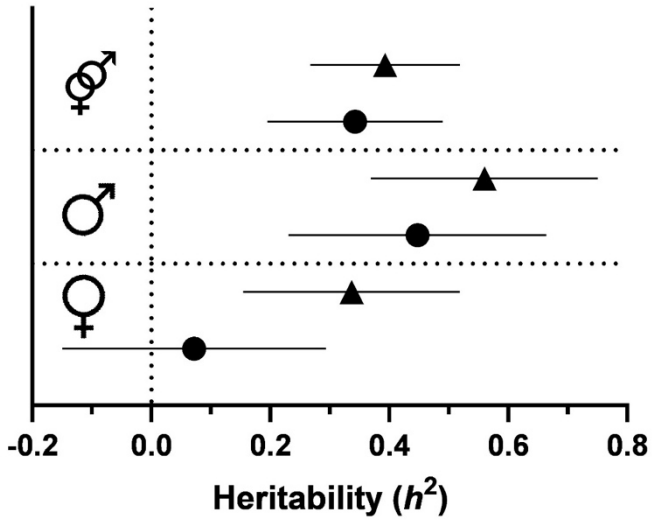


**Figure 4.** Heritability estimates (± s.e.m) of pied flycatcher telomere length based on parent-offspring regression (mid-parent [*i.e.* average of paternal and maternal telomere length], paternal & maternal) at chick days 5 (triangles) and 12 (circles). Sample sizes [for regression: chick day 5/chick day 12] are: Mid-parent: 113/104: Paternal: 117/108; Maternal: 132/115.

Using mid parent-offspring regressions, there was no clear indication that heritability of telomere length differed between populations (Population × Mid-parent: TL day 5: $F_{5, 92.05} = 1.33$, $p = 0.26$; day 12: $F_{5, 28.9} = 0.47$, $p = 0.80$). Yet, mid-parent-offspring regressions suggest a moderate heritability of telomere length across pied flycatcher populations, with a $h^2 = 0.40$ for telomere length at day 5 ($F_{1, 105.2} = 9.75$, $p = 0.002$) and $h^2 = 0.34$ for telomere length at day 12 ($F_{1, 96.04} = 5.4$, $p = 0.02$; Fig. 4). While father-offspring regressions suggest a moderate to high heritability of telomere length (day 5: $h^2 = 0.56$, $F_{1, 109.9} = 8.59$, p = 0.004; day 12: $h^2 = 0.45$, $F_{1, 93.42}$ = , p = 0.04; Fig. 4), mother-offspring regressions show lower non-significant heritability estimates (day 5: $h^2 = 0.34$, $F_{1, 112.8} = 3.42$, p = 0.07; day 12: $h^2 = 0.07$, $F_{1, 66.23} = 0.11$, p = 0.75; Fig. 4).

Although non-significant, there was some indication that the relationship between paternal telomere length and offspring day 5 telomere length differed between populations (Population × Paternal TL: $F_{5, 97.67} = 2.12$, $p = 0.07$). Running regressions with chick TL at day 5 and paternal TL for each population separately suggested high heritability in England (East Dartmoor) and Estonia (Kilingi-Nõmme), moderate heritability in La Hiruela population in Spain and northern Finland (Oulu), and null heritability in southern Finland (Turku) and Valsaín population in Spain (Fig. 5).

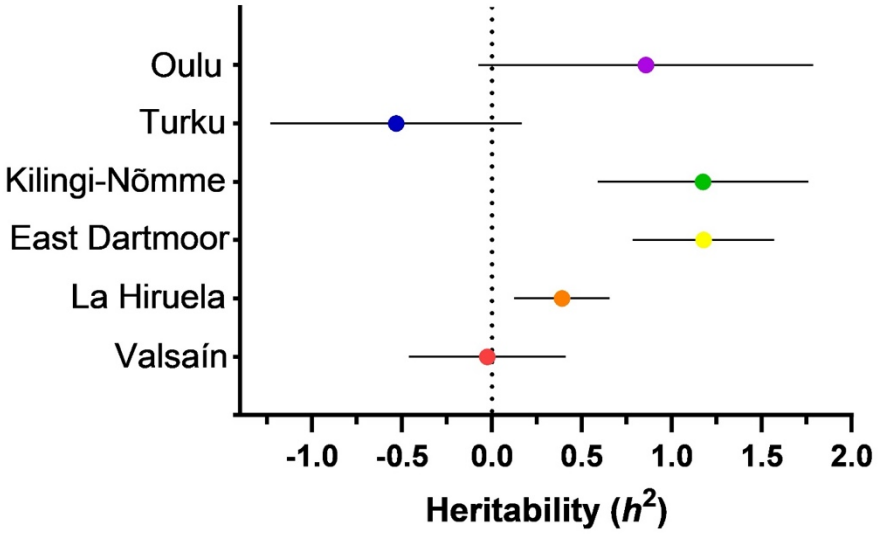


**Figure 5.** Heritability (± s.e.m) estimates between pied flycatcher father and offspring telomere length at day 5 in six different populations across Europe. Sample sizes [for Population] are: Oulu, Finland: 18; Turku, Finland: 16; Kilingi-Nõmme, Estonia: 18; East Dartmoor, England: 21; La Hiruela, Spain: 21; Valsaín, Spain: 23.

## Discussion

We found consistent variation in telomere length across European pied flycatcher populations and across different life stages (*i.e.* soon after hatching, close to fledging and in adulthood). There was no clear support for a relationship between migration distance and telomere length across populations. There was some indication that the rate of early-life telomere shortening varies between populations, but this effect was less pronounced than the pattern observed for chick body mass and growth rate. Heavier chicks had shorter telomeres in the early nestling period across all populations, an effect that was similar in direction but weaker close to fledging age. Interestingly, early-life growth rate was related to early-life telomere shortening rate, but in a population-dependent manner, with only northern populations exhibiting more telomere shortening when growing fast. While heritability of telomere length based on mid-parent-offspring regression was moderate ($h^2$ = 0.34 - 0.40), heritability estimates did not differ between populations. However, analysing heritability separately for fathers and mothers suggested moderate to high paternal heritability of telomere length, but weaker, non-significant maternal heritability of telomere length. Furthermore, there was some evidence that the level of the paternal heritability of telomere length differs between populations.

### *Telomere dynamics across populations*

As expected, telomeres shortened gradually both during early-life (-11.7%) and between fledging and adulthood (-12.6 %). We found no evidence for consistent telomere lengthening in any

population. The overall dynamics observed with age did not differ between populations, despite variation in environmental conditions experienced across the North-South breeding range (Lundberg & Alatalo, 1992; Samplonius et al., 2018). Yet, there were clear differences in telomere length between populations. English birds (East Dartmoor) had the longest telomeres followed by Spanish birds (Valsaín and La Hiruela) having similar telomere lengths while the Estonian and Finnish birds (Kilingi-Nõmme, Turku, and Oulu) had the shortest telomeres. Notably, telomeres at the population level did not shorten with increasing migration distance as birds breeding in the mid longitudinal part of the breeding range (England, East Dartmoor) had longer telomeres at any stage than the birds breeding further south. Furthermore, the pattern between migration distance and telomere length was similar in chicks at day 12. Our sample of different populations was clearly limited for making strong conclusions of this relationship. However, the English birds having the longest telomeres and the pattern of telomere change between nestling and adult stages being similar in all the populations indicates that the absolute differences in telomere length among populations are more attributable to other factors than to differences in migration distance. Previous studies have associated migratory lifestyle with shorter telomeres in dark-eyed juncos (*Junco hyemalis*) and longer migration distance with reduced fitness and survival in sanderlings (*Calidris alba*) (Bauer et al., 2016; Reneerkens et al., 2020). However, these associations might be more attributable to subspecies differences (Bauer et al., 2016) and varying environmental conditions across distinct wintering sites (Reneerkens et al., 2020) rather than the migration distance *per se*. Nevertheless, due to logistical difficulties, our study is missing the pied flycatchers with the longest migration distance (breeding in Siberia) that could have been truly informative regarding this question. Closer examination of other potential factors affecting population telomere length and inclusion of more populations are therefore needed to further ascertain our results.

In this study, population telomere length was not linearly associated with migration distance. However, as there are genetic differences between pied flycatcher populations (Lehtonen et al., 2012; Lehtonen et al., 2009), we cannot exclude that the length of migration and telomere length are genetically correlated at the population level, as is potentially the case in dark-eyed juncos (Bauer et al., 2016). Additionally, chromosomes contain non-terminal telomeric repeat sequences (interstitial telomeres, ITS) that are included in the relative telomere length measure (Foote, Vleck, & Vleck, 2013). Amounts of ITS might differ between populations, which could explain why telomere length, but not shortening rate, differed markedly between populations. The longest telomeres of the English population (East Dartmoor) might also be explained by environmental differences between the populations breeding sites, with East Dartmoor being Sessile oak (*Quercus petraea*) dominated deciduous forest. Deciduous forests of central Europe might be more favourable for breeding than northern, conifer-dominated forests or southern mountainous forests, as indicated by bigger clutches in central Europe compared to northern and southern populations (Sanz, 1997), and a higher high-quality prey abundance in deciduous forests (Burger et al., 2012). Furthermore, egg

yolk carotenoid levels are highest in central European pied flycatcher populations relative to southern and northern populations, although a population in Spain showed high concentrations of a few carotenoids (Eeva et al., 2011). Carotenoid concentrations in the eggs are reflective of female diet during egg laying (Török et al., 2007), and thus can be an indicator of environmental quality. Also, carotenoids work as antioxidants that alleviate oxidative stress (Surai, Fisinin, & Karadas, 2016) and possibly telomere shortening (Kim & Velando, 2015; Pineda-Pampliega et al., 2020). Therefore, possible higher levels of carotenoids in the diet of English (East Dartmoor) birds might contribute to the telomere length differences between populations we observed. High predator abundance and/or parasite prevalence can have a negative effect on telomeres (Asghar et al., 2015; Kärkkäinen et al., 2019), and thus unequal exposures to predators and parasite might also cause between-population differences in telomere dynamics. However, these are unlikely explanations for our results, although there is geographical variation in abundance of both predators and blood parasites of passerine birds in Europe (Díaz et al., 2013; Scheuerlein & Ricklefs, 2004). Predator abundance decreases with increasing latitude (Díaz et al., 2013) while we observed that birds from northern latitudes (Estonia and Finland) had the shortest telomeres, which is the opposite of the expected predator effect. Similarly, prevalence of certain blood parasites were lowest in southern latitudes increasing with increasing latitude (Scheuerlein & Ricklefs, 2004) but we observed the English birds to have longer telomeres than the Spanish birds.

*Early-life telomere dynamics and growth*

The rate of telomere shortening within chicks differed between populations, but was not consistent along the north-south gradient, as the chicks from southern Finland (Turku), England (East Dartmoor), and one Spanish population (Valsaín) tended to have higher rates of telomere shortening than chicks from northern Finland (Oulu), Estonia (Kilingi-Nõmme), and the other Spanish population (La Hiruela). Although we did not measure any environmental variables, such differences in early-life telomere shortening could reflect local differences in environmental factors *e.g.* forest type. However, the observed differences might simply reflect the local breeding conditions of the year as our data was collected over a single breeding season. Thus, more research is needed to evaluate the potential geographical variation in early-life telomere shortening and its underlying factors.

Differences in chick growth between populations were clearer than differences in telomere shortening. We found that chicks from Spanish populations were lighter at day 5 but showed the highest growth rates from day 5 to day 12, and eventually matched the masses of chicks from other populations by day 12. This later growth peak in the Spanish flycatchers might be explained by elevation differences between populations. While all other populations in this study were at relatively low elevations (10-300 m above sea level), the Spanish flycatchers breed around 1 200 m above sea level. A previous study demonstrated great

431 tit (*Parus major*) chicks, a species commonly breeding at low elevations, showed slower growth at high
432 elevations (Stier et al., 2016), a difference potentially explained by the changes in prey availability, *i.e.* insect
433 communities with increasing elevation (Hodkinson, 2005). Across populations, at day 5, heavier chicks had
434 shorter telomeres, and the tendency was the same at day 12. Previous studies have also associated fast
435 growth with faster telomere shortening (Monaghan & Ozanne, 2018; Stier, Hsu, et al., 2020; Tarry-Adkins,
436 Martin-Gronert, Chen, Cripps, & Ozanne, 2008), as growth requires pronounced metabolic activity and
437 cellular proliferation (Monaghan & Ozanne, 2018). Interestingly, chick growth more negatively affected
438 telomere shortening rate at higher latitudes (Finland and Estonia) than at southern latitudes (England and
439 Spain). To our knowledge, this sort of pattern has not been observed previously. Chicks growing in mostly
440 conifer-dominated forests further north might suffer from low-quality food (Burger et al., 2012), which
441 together with the metabolic and oxidative stress caused by somatic growth could be detrimental for telomere
442 maintenance. Additionally, carotenoids found in the eggs at mid latitudes, but also to some extent in
443 southern Europe pied flycatcher populations (Eeva et al., 2011), might better safeguard chick telomeres as
444 they grow (Min & Min, 2017; Pineda-Pampliega et al., 2020).

445

446 *Heritability of telomere length in the pied flycatcher*

447 Heritability estimates for telomere length range from 0 to 1 in wild populations (Dugdale &
448 Richardson, 2018). Based on mid-parent–offspring regressions, we found the heritability of telomere length
449 to be moderate to high ($h^2$ = 0.34 – 0.40) in the pied flycatcher. We also found that the heritability was higher
450 at chick day 5 than chick day 12, suggesting that the resemblance of offspring and parent telomere length
451 diminishes with age due to environmental effects during growth, similarly to that observed in wild king
452 penguins (*Aptenodytes patagonicus*) (Reichert et al., 2015). Several previous bird studies have reported
453 maternal telomere length inheritance, for example in kakapo (*Strigops habroptilus*), king penguin, great
454 reed warbler (*Acrocephalus arundinaceus*), and jackdaw (*Corvus monedula*) (Asghar, Bensch, Tarka, Hansson,
455 & Hasselquist, 2015; Bauch, Boonekamp, et al., 2020; Horn et al., 2011; Reichert et al., 2015). Recently, some
456 studies have identified stronger paternal than maternal effect on chick telomere length (Bennett et al., 2021;
457 Bouwhuis, Verhulst, Bauch, & Vedder, 2018). In our study, the higher paternal than maternal heritability is
458 quite surprising considering frequent occurrence of extra-pair paternity in pied flycatchers that would lower
459 the heritability estimates (Alatalo, Gustafson, & Lundberg, 1989). However, while some studies have
460 estimated heritability based on 'animal models' methodology that can separate genetic and environmental
461 effects to some extent (Asghar et al., 2015; Bauch, Boonekamp, et al., 2020), we used parent-offspring
462 regressions and thus were unable to separate the environmental effects from the genetic effects.
463 Interestingly, stronger associations between offspring telomere length and foster, but not biological mother

464 telomere length were found for the king penguin (Viblanc et al., 2020), suggesting the importance of parental
465 care for offspring early-life telomere length. Accordingly, a study cross-fostering chicks of the collared
466 flycatcher (*Ficedula albicollis*), a close relative of the pied flycatcher, shows the heritability of telomere length
467 to be much lower ($h^2$ = 0.09) than in this study (Voillemot et al., 2012). Furthermore, a study on pied
468 flycatchers showed that while feeding effort of females remained constant with altered brood sizes, males
469 modified their feeding rates according to the demand of the brood (Moreno, Cowie, Sanz, & Williams, 1995),
470 which could turn into the observed stronger paternal effect on chick telomere length during early life.
471 Therefore, it is possible that our heritability estimates reflect more early-life parental effects and/or shared
472 environmental effects than genetic inheritance (Dugdale & Richardson, 2018). Indeed, we found the
473 associations between early-life telomere length and paternal telomere length to be stronger in northern
474 Finland (Oulu), Estonia (Kilingi-Nõmme), and England (East Dartmoor) than other populations, possibly
475 indicating differences in the parental care, environmental conditions and/or levels of extra-pair paternity. It
476 is also good to acknowledge that assortative mating can bias single-parent heritabilities (Merilä & Sheldon,
477 2001). In birds, assortative mating for telomere length has been observed before in barn swallows (*Hirundo*
478 *rustica*) and king penguins (Khoriauli et al., 2017; Schull et al., 2018), but to what extent this happens in pied
479 flycatchers remains to be investigated.

480

481 *Conclusion*

482 To our knowledge, we provide the first study assessing large-scale geographical population
483 differences in telomere length and dynamics. Our results show that across a latitudinal gradient, European
484 pied flycatcher populations exhibit differences in mean telomere length both in chicks and adults that likely
485 reflect regional environmental conditions or genetic differences. These marked population differences in
486 telomere length dispute the common practice of using 'species' as unit in meta- and comparative analyses,
487 as recently suggested by Canestrelli et al. (2020) and highlight the need to study telomeres at between-
488 population level. Future studies would benefit from closer examination of potential factors driving the
489 observed between-population differences, and from assessing whether these differences in telomere length
490 translate into between-population differences in lifespan, survival, and/or fitness proxies.

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**Ethics**

The collection of blood samples in Finland was licenced by the Animal Experiment Board in Finland (authorization license ESAVI/3021/04), in Estonia by the Animal Procedures Committee (licence no. 107) of the Estonian Ministry of Agriculture, in England by the Home Office (PPL 3003283), and in Spain by the Bioethics Committee of CSIC and Comunidad de Madrid (Valsaín: Ref. PROEX 128/19; La Hiruela: 10/106650.9/19) and Castilla-La Mancha (PR-21-2017).

**Author contribution**

TK, AS and TL conceived the study following an original idea of JM. TK, MB, AC, JMP, JP, JM, RLT, and VT collected the blood samples. TK conducted the laboratory analyses with AS assistance. TK and AS analyzed the data. TK and AS wrote the manuscript with input from TL and other co-authors.

**Conflict of interest**

The authors declare no conflicts of interest.

**Data accessibility**

Data used in this study will be made publicly available in Figshare upon acceptance.

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**Supplementary information to:**

# Population differences in the length, early-life dynamics, and heritability of telomeres among European pied flycatchers

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**Figure S1.** Associations between migration distance (km) and relative telomere length (mean) in the pied flycatcher fledglings (12 days after hatching; circles) and adults (averaged breeding pair; squares). Standard errors of the means (± sem) have been added to illustrate the population variation in telomere length. Fledgling values (circles) have been moved slightly to the right to clarify the error bars. Populations from the shortest migration distance to the longest: Spain (average of Valsaín and La Hiruela, red), England (East Dartmoor, yellow), Estonia (Kilingi-Nõmme, green), southern Finland (Turku, blue), and northern Finland (Oulu, purple).

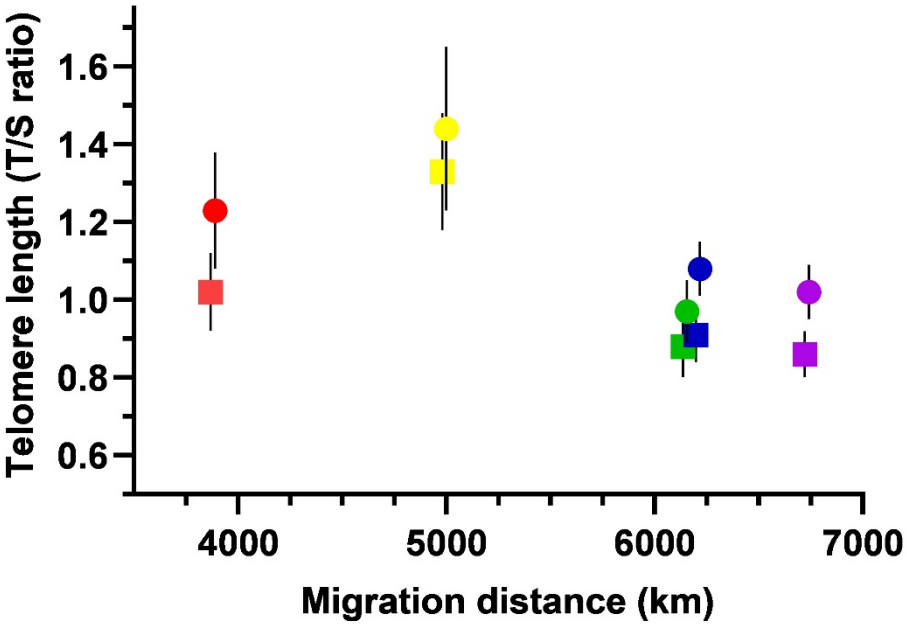
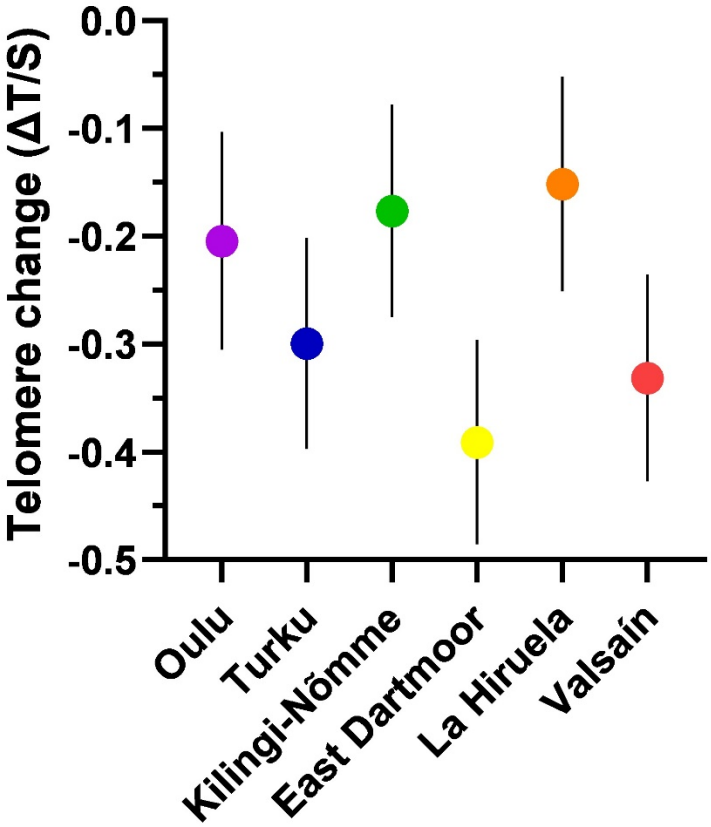
**Figure S2.** Change in relative telomere length during nestling period in the pied flycatcher (Δ telomere length between days 12 and 5) in six populations across a north-south gradient in Europe. The effect of population was marginally significant ($p$ = 0.07) in explaining variation in early-life telomere change (see results for details). Telomere change values were corrected as recommended by Verhulst et al. (2013), and to obtain more intuitive estimates for the corrected telomere change values, the difference of the averages of first and second measurement was added to each telomere change value. Values are estimated marginal means ± s.e.m. Sample sizes [for Population] are: Oulu, Finland: 17; Turku, Finland: 18; Kilingi-Nõmme, Estonia: 19; East Dartmoor, England: 21; La Hiruela, Spain: 18; Valsaín, Spain: 21.