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RESEARCH ARTICLE

Intraspecific Variation in Evolution and Ecology

Among-population variation in telomere regulatory proteins and their potential role as hidden drivers of intraspecific variation in life history

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Abstract

1. Biologists aim to explain patterns of growth, reproduction and ageing that characterize life histories, yet we are just beginning to understand the proximate mechanisms that generate this diversity. Existing research in this area has focused on telomeres but has generally overlooked the telomere's most direct mediator, the shelterin protein complex. Shelterin proteins physically interact with the telomere to shape its shortening and repair. They also regulate metabolism and immune function, suggesting a potential role in life history variation in the wild. However, research on shelterin proteins is uncommon outside of biomolecular work.
2. Intraspecific analyses can play an important role in resolving these unknowns because they reveal subtle variation in life history within and among populations. Here, we assessed ecogeographic variation in shelterin protein abundance across eight populations of tree swallow (*Tachycineta bicolor*) with previously documented variation in environmental and life history traits. Using the blood gene expression of four shelterin proteins in 12-day-old nestlings, we tested the hypothesis that shelterin protein gene expression varies latitudinally and in relation to both telomere length and life history.
3. Shelterin protein gene expression differed among populations and tracked nonlinear variation in latitude: nestlings from mid-latitudes expressed nearly double the shelterin mRNA on average than those at more northern and southern sites. However, telomere length was not significantly related to latitude.

4. We next assessed whether telomere length and shelterin protein gene expression correlate with 12-day-old body mass and wing length, two proxies of nestling growth linked to future fecundity and survival. We found that body mass and wing length correlated more strongly (and significantly) with shelterin protein gene expression than with telomere length.
5. These results highlight telomere regulatory shelterin proteins as potential mediators of life history variation among populations. Together with existing research linking shelterin proteins and life history variation within populations, these eco-geographic patterns underscore the need for continued integration of ecology, evolution and telomere biology, which together will advance understanding of the drivers of life history variation in nature.

KEYWORDS
bird, latitude, life history, POT1, shelterin proteins, telomere, TPP1, TRF2

1 | INTRODUCTION

Evolutionary biologists aim to explain diversity in patterns of growth, reproduction and ageing that characterize life histories (sensu Stearns, 1992). Life history traits often vary predictably with geography and ecology (Gaston et al., 2008). However, many eco-geographic rules do not fully address the proximate mechanisms underlying variation in life history, despite repeated calls to integrate physiological mechanisms into life history theory (Ricklefs & Wikelski, 2002).

Life history traits are often linked to telomeres (Monaghan, 2010), the chromosomal structures that preserve genomic integrity and shorten over time (Blackburn, 1991; Remot et al., 2021; Young, 2018), yet the telomere's protective shelterin proteins are often ignored. The shelterin complex includes six proteins (de Lange, 2018; Myler et al., 2021; Figure 1): TRF1 and TRF2 bind double-stranded telomeric repeats; RAP1 associates with TRF2; and TIN2 physically links TRF1 and TRF2 with TPP1, which recruits POT1. Together, this complex forms a protective telomere cap that negatively regulates telomere accessibility by telomerase, the enzyme that repairs telomere length (de Lange, 2018). Critically, telomere loss and other factors may free up shelterin proteins to act away from the telomere end (Mukherjee et al., 2018, 2019), where they may influence life history via transcriptional regulation of metabolic and immune function (Akincilar et al., 2021; Wolf & Shalev, 2023; Ye et al., 2014). This provides a putative mechanism by which stress-induced and ecogeographic variation in telomere loss (Chatelain et al., 2020; Karkkainen et al., 2022; Stier et al., 2016) may causally contribute to life history (e.g. survival, lifespan: Heidinger et al., 2012; Wilbourn et al., 2018).

Shelterin proteins could expand the causal links between telomere biology and ecologically relevant phenotypes, but only a few recent studies have focused on shelterin in nature. For example, some shelterin proteins are more highly expressed in mammalian species with longer lifespans (e.g. TIN2, TRF1; Ma et al., 2016; MacRae et al., 2015). At the intraspecific level, decreases in shelterin

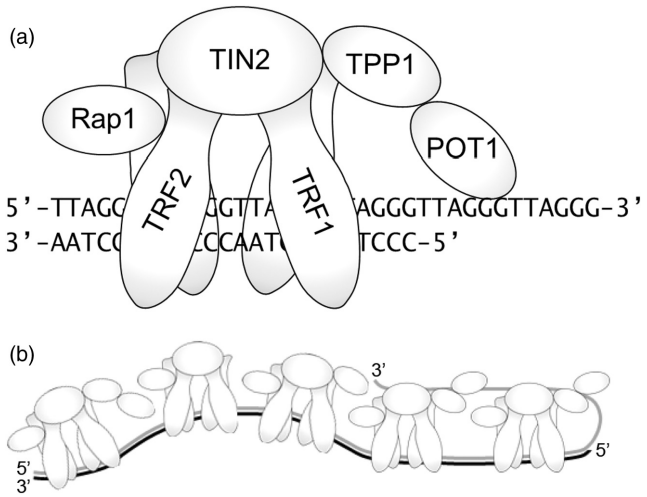

FIGURE 1 Schematic of the shelterin protein complex. (a) The six-subunit shelterin protein complex binds to double-stranded and single-stranded telomeric DNA (TTAGGG repeats). (b) Shelterin complexes bind repeatedly along the telomere's length. Co-factors are not shown.

occur in response to natural stressors and are linked to survival (Rouan et al., 2021; Wolf et al., 2022), suggesting that low shelterin may be adaptive. In addition, Wolf et al. (2022) showed that shelterin POT1 gene expression outperformed telomere length in predicting fitness-related traits within a population of wild birds. These intraspecific approaches are key to assessing subtle variation occurring among populations without confounding species effects. On the other hand, biomedical work suggests that increases in shelterin may provide temporarily heightened DNA protection during aerobic stress (sensu Ludlow et al., 2013). Extremely high or low shelterin can also promote senescence or immortalization of cancer cells (Akincilar et al., 2021). Together, these observations suggest that telomere length and, by extension, telomere regulation may be shaped by stabilizing selection. Altogether, it remains unclear how

subtle natural variation in shelterin protein abundance contributes to life history.

The first step to integrating shelterin proteins into a life history framework is to quantify their standing variation in the wild and assess correlations with ecology and life history. In our study, we used free-living tree swallows (*Tachycineta bicolor*). Tree swallows migrate north each spring from the southernmost United States, Central America and Caribbean to breeding grounds ranging from Alaska to the mid-southern United States (Winkler et al., 2020), although this range has been expanding *south*, for example, into South Carolina and Alabama in the last three decades (Shutler et al., 2012; Wright et al., 2019). Previous work shows life history variation among populations: birds breeding at higher latitudes have shorter breeding seasons, slightly larger clutches and higher mortality rates (Ardia, 2005; Dunn et al., 2000; Winkler et al., 2020), which may be driven in part by migration routes and glucocorticoid levels (Gow et al., 2019; Zimmer et al., 2020; but see Siefferman et al., 2023). Critically, this range also varies in environmental factors, such as local food availability and nest temperatures (Ardia, 2006; Zimmer et al., 2020).

We capitalized on this range to assess the spatial variation of shelterin protein abundance in nestling tree swallows across eight populations in the eastern United States. We expected that shelterin protein gene expression would vary latitudinally and in relation to proxies of life history. Based on findings that long-lived species have higher shelterin protein abundance (e.g. Ma et al., 2016; MacRae et al., 2015), one prediction is that more southerly populations, which have a slower life history strategy, will express more shelterin. Alternatively, populations with slower life histories may express less shelterin, if insights gained from within-population analyses (e.g. Wolf et al., 2022) apply across larger spatial scales. We also assessed associations of shelterin proteins with telomere length and age-specific body size, an established proxy of nestling growth. We predicted that shelterin proteins would better predict nestling body size than telomere length, given that shelterin proteins regulate metabolism. While little is known about the consequences of shelterin proteins at the organismal scale (but see above), they should nevertheless vary with life history and the environment. Documenting intraspecific variation in shelterin levels and their covariation with fitness-related traits is foundational to future research on the proximate and ultimate outcomes of shelterin protein expression, and may reveal a novel mechanism contributing to life history and ageing.

2 | MATERIALS AND METHODS

2.1 | Study populations

Data were collected from eight populations in the eastern United States, spanning nearly 10 degrees of latitude (Table 1; Figure 2a): Ithaca, New York (42.28°N, 76.29°W); Amherst, Massachusetts (42.22°N, 72.31°W); Linesville, Pennsylvania (41.65°N, 80.43°W); Bloomington, Indiana (39.17°N, 86.53°W); Lexington, Kentucky (38.11°N, 84.49°W); Knoxville, Tennessee (35.90°N, 83.96°W); Davidson, North Carolina (35.53°N, 80.88°W); and Santee, South Carolina (33.49°N, 80.36°W). These populations do not represent the entire breeding range of this species and, in particular, do not extend to the northern edge in Canada and Alaska. All methods were approved by institutional IACUCs (Cornell University #2019-0023; Amherst College #201-4; Indiana University #18-004; University of Kentucky #2015-2003) and Master Bird Banding Permits #23968, #24118, #22183 and #24129.

2.2 | Sampling of nestlings

Nest boxes were monitored for hatch dates, but in cases where hatch dates were missed (e.g. due to weather or COVID-related staffing shortages), hatch dates were estimated using existing growth curves (McCarty, 2001; Wolf et al., 2021) and accounted for in all statistical analyses. Data from multiple populations shows that the average peak of postnatal growth occurs around 6 days old (McCarty, 2001;

TABLE 1 Sample sizes by state for each model.

	Year(s)	Avg age (days ± SE)	Count female	Count male	Mass	Wing length	Telomere length	Shelterin pc1
NY	2020	12.00 ± 0.00	15	17	32	32	32	0[a]
MA	2020	12.10 ± 0.06	24	14	38	39	39	19
PA	2020	12.10 ± 0.05	20	16	36	36	36	36
IN	2019, 2020[b]	12.00 ± 0.02	32	28	60	60	41	18
KY	2020	12.00 ± 0.00	16	9	25	0	25	0
TN	2020	12.00 ± 0.10	16	16	32	32	32	29
NC	2020	11.80 ± 0.09	22	11	33	33	33	33
SC	2020–2021	12.10 ± 0.09	11	12	23	23	23	33
		Total count	156	123	279	255	261	168

Note: Multiple nestlings were measured per nest, but only one median-massed nestling was selected a priori for all analyses. For logistical reasons, not all samples were collected in the same year. Note that ([a]) NY RNA samples were excluded, and ([b]) shelterin pc1 and telomere length were measured using two separate cohorts of IN nestlings (2019, 2020, respectively).

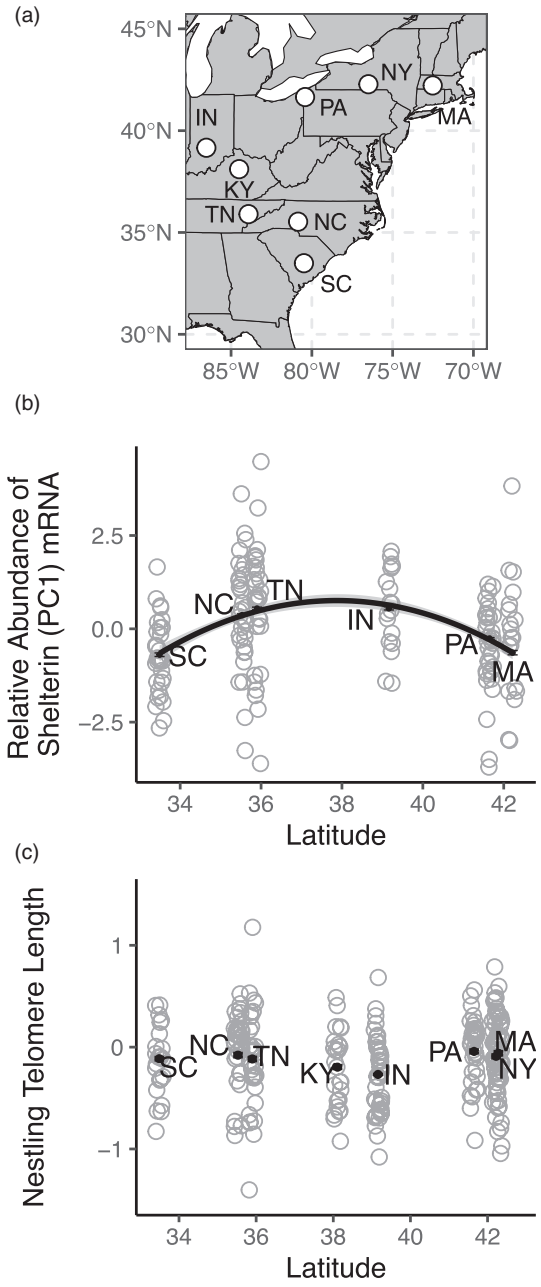

FIGURE 2 Latitudinal variation in telomere biology in the blood of nestling tree swallows (a) across a latitudinal gradient in the eastern US. (b) Model outputs for shelterin protein gene expression (condensed into PC1). One unit of PC1 equates to increases in gene expression of 50% for POT1 and 100% (or doubling) for TPP1, TRF2 and TRF2IP. (c) Model outputs for relative telomere length (T/S ratio). Points and grey circles represent average ± SE values per population and individual points, respectively. Shaded areas show 95% confidence intervals.

Wolf et al., 2021). Growth then slows and plateaus near adult size by 12 days old, just as feather development accelerates. We targeted 12-day-old nestlings because they have just completed the rapid period of postnatal growth. Many studies therefore use morphological data at this critical time period as a proxy of nestling growth (Gebhardt-Henrich & Richner, 1998; Haywood & Perrins, 1992; Magrath, 1991; Martin et al., 2018; McCarty, 2001). Population variation in growth rates occurs primarily after peak growth but does not map neatly onto latitude, at least not in the northern (historical) range where previous research has been focused (Ardia, 2006; McCarty, 2001).

We sampled nestlings at 12.03 ± 0.01 days old (hatch day = day 1, range = 10–14 days). We sampled ~30 nests per population (Table 1), though logistical constraints prevented the collection of RNA in Kentucky. Upon arrival at each nest, we immediately collected whole blood from the brachial vein of 2–3 nestlings per nest (≤200 μL, below the maximum suggested volume based on body mass; Gaunt et al., 1997), and we avoided obvious runts with atypical growth. We collected blood in separate tubes for DNA and RNA analyses. We banded nestlings with a USGS band and weighed them to the nearest 0.1 g. We also measured flattened wing length using a wing ruler. We stored blood on ice or dry ice in the field, and later stored it at −80°C.

Due to limited budgets, we made the decision a priori to conduct laboratory analyses for a single nestling per nest. When possible, we selected the nestling with the median mass. If the median-massed nestling was not bled or failed to produce a sufficient blood sample, we selected the nestling with the closest mass to the median. In nests with even brood sizes, we randomly selected one of the two nestlings with median mass for telomere and gene expression analyses. In all states except Indiana, telomere length and gene expression data come from the same individual.

2.3 | qPCR for telomere length

We extracted DNA from whole blood (following Wolf et al., 2022) and used primers *telc* and *telg* (adapted from Cawthon, 2009) to quantify telomere length relative to the single-copy gene GAPDH. Samples were run in triplicate, and mean values were used to calculate the T/S ratio of telomere repeat copy number (T) to our single gene copy number (S) using the formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})_{\text{reference}} - (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})_{\text{sample}}$. The intraclass correlation coefficient (ICC) for intraplate repeatability was 0.951 ± 0.03 (95% CI = 0.944, 0.957) for GAPDH C_t values and 0.926 ± 0.09 (95% CI = 0.916, 0.935) for telomere C_t values. The ICCs for interplate repeatability were 0.96 ± 0.03 (95% CI = 0.87, 0.98) for GAPDH C_t values, 0.89 ± 0.06 (95% CI = 0.73, 0.95) for telomere C_t values and 0.79 ± 0.10 (95% CI: 0.54–0.90) for the T/S ratio (based on $2^{-\Delta Ct}$ values). Plates ($n = 13$) were balanced by population, sex, relative date of sampling within each population and brood size.

2.4 | Nestling sexing protocol

Nestlings were molecularly sexed using DNA following established methods (Griffiths et al., 1998; Wolf et al., 2022).

2.5 | Shelterin protein primer design

Shelterin proteins are relatively conserved across taxa (de Lange, 2018; Myler et al., 2021), and earlier work has identified at least four shelterin proteins in the chicken (De Rycker et al., 2003; Konrad et al., 1999; Tan et al., 2003; Wei & Price, 2004). Our shelterin protein primer sets were developed using the tree swallow transcriptome (accession #GSE126210; Bentz et al., 2019). TRF2 exhibits multiple variants in passerines, and a BLAST search confirmed that our primer set targets TRF2 in closely related barn swallows (*Hirundo rustico*). TPP1 and POT1 each have a single transcript in adult tree swallows that is highly expressed across tissues, and BLAST searches confirmed that our primer sets targeted TPP1 and POT1 genes, respectively, in multiple bird species. We also designed primers for RAP1 based on tree swallow transcripts of TRF2IP (TRF2-interacting protein), a common alias for RAP1. However, this study omits TRF1 due to negligible expression in nestling blood and TIN2 because we could not confidently identify the passerine sequence for TIN2. Thus, we quantified gene expression for four key components of the shelterin complex: TRF2, RAP1, TPP1 and POT1 (primer sequences in Table S1).

2.6 | qPCR for Shelterin protein gene expression

We extracted RNA using a phenol-chloroform-based Trizol method (Invitrogen, Carlsbad, CA) with PhaseLock tubes (5PRIME, #2302830). We synthesized cDNA using 1 μg RNA and Superscript III reverse transcriptase (Invitrogen), treated with DNAase (Promega, Madison, WI) and RNase inhibitor (RNAsin N2111, Promega). For each gene of interest, we used the $2^{-\Delta\Delta Ct}$ method of quantification (Livak & Schmittgen, 2001), in which expression is normalized to the geometric mean C_t of two reference genes for each sample (Vandesompele et al., 2002) and relative to a calibrator sample on each plate. Reference genes correct for technical variation in cDNA quantity across samples and, as such, must (i) be highly expressed, (ii) exhibit low variability among samples and (iii) show no significant variation among biological categories of interest. Our reference genes were PPIA (peptidylprolyl isomerase A; Virgin & Rosvall, 2018) and MRPS25 (Mitochondrial Ribosomal Protein S25; Woodruff et al., 2022). Preliminary work showed that New York samples exhibited markedly higher gene expression of these and a third reference gene (GAPDH). This violates assumption (iii) of the $2^{-\Delta\Delta CT}$ method, and we therefore had to omit New York gene expression data. The remaining six populations exhibited limited among-population variation in reference gene expression (non-significant state differences or ≤0.5 C_t of the study-wide average).

Samples were run in triplicate alongside No Template Controls (NTCs) using PerfeCta SYBR Green FastMix with low ROX (Quanta Biosciences, Gaithersburg MD) on 384-well plates using an ABI Quantstudio 5 machine with Quantstudio Design & Analysis software (v1.4.3, Thermo Fisher Scientific, Foster City, CA). Each well included 3 μL of cDNA diluted 1:50 (or 3 μL water for NTCs) and primers diluted to 0.3 μM in a total volume of 10 μL. All reactions use the following thermal profile: 10 min at 95°, followed by 40 cycles of 30 s at 95°, 1 min at 60° and 30 s at 70°, with a final dissociation phase (1 min at 95°, 30 s at 55° and 30 s at 95°) that confirmed single-product specificity for all samples. All samples fell within the bounds of the standard curve, and reaction efficiencies were within 100 ± 15%. Each gene was run on 1.5 plates, balanced by population. Intraclass correlation coefficients for triplicates were 0.996 ± 0.01 (95% CI = 0.995, 0.997) for PPIA C_t values, 0.994 ± 0.009 (95% CI = 0.993, 0.996) for MRPS25 C_t values, 0.975 ± 0.05 (95% CI = 0.967, 0.982) for POT1 C_t values, 0.940 ± 0.05 (95% CI = 0.923, 0.954) for TRF2 C_t values, 0.975 ± 0.05 (95% CI = 0.968, 0.981) for TRF2IP C_t values and 0.996 ± 0.007 (95% CI = 0.995, 0.997) for TPP1 C_t values.

2.7 | Statistical analyses

All analyses were performed in R (version 3.5.3, RStudio Team, 2019). We fitted general linear mixed effects models using the *nlme* package (Pinheiro et al., 2017) to perform two main types of analyses (below).

All four shelterin proteins were positively correlated (log(log2-transformed gene expression), $0.21 < R < 0.61$, Figure S1), so we used principal components analysis to reduce the dimensionality of these data. PC1 had an eigenvalue of 1.52, accounting for 58% of the total variance. PC1 positively loaded for all four shelterin proteins (TRF2: 0.59, TRF2IP: 0.51, TPP1: 0.45 and POT1: 0.44). Based on these loadings and the fact that 1 unit of log2 space denotes a doubling of abundance, we can infer that 1 additional unit of PC1 equates to increases in gene expression of 50% for POT1 and 100% (or doubling) for TPP1, TRF2 and TRF2IP.

To test for ecogeographic trait variation, we ran separate Gaussian models for body mass, wing length, log-transformed telomere length and shelterin protein gene expression. While latitude was our main fixed effect, we also included latitude^2 for several reasons. First, populations closer to the range edge may have unique physiological traits that alter telomere dynamics and life history, including immune function and growth (Chatelain et al., 2020; Martin et al., 2014; Myles-Gonzalez et al., 2015). This may be relevant to the South Carolina population, which is near the southward expansion edge of breeding range (Shutler et al., 2012; Wright et al., 2019). Such 'pioneers' may have unique phenotypes (Siefferman et al., 2023). Even without individual variation driven by range expansions, nonlinear patterns with latitude can emerge from spatial contrast in selection by abiotic and biotic factors (MacArthur, 1984; Paquette & Hargreaves, 2021). Each model included latitude, latitude^2, sex, age at sampling and ageing method (i.e. known or estimated age) as fixed effects. Brood size was also included as a fixed effect, as it may influence traits of interest and exhibits subtle latitudinal variation in previous work (Dunn et al., 2000). We did not detect multicollinearity among this group of fixed effects (variable inflation factors ≤2). Population was included as a random effect to account for multiple birds sampled at each site. In addition, models of telomere length

and shelterin gene expression included random effects of qPCR plate. Note that results for these models run with qPCR plate as a *fixed* effect are equivalent to those in which qPCR plate was included as a *random* effect (Table S2). One outlier was removed (using Grubbs test) for models predicting body mass and PC1 for shelterin protein gene expression.

To evaluate whether shelterin protein gene expression or telomere length better predicts nestling morphology, we use an information-theoretic approach. For model comparisons predicting body mass and wing length, we created a three-model set. The null model included known or likely predictors of body mass or size: latitude, latitude^2, nestling age at sampling, ageing method, sex and brood size as fixed effects, with population as a random effect. The remaining two models additionally contained either shelterin protein gene expression or log-transformed telomere length, allowing us to evaluate the prediction that shelterin proteins better predict morphology than telomere length. As above, we did not detect multicollinearity among fixed effects for any candidate model (variable inflation factors ≤2). We used Akaike information criterion (AIC_c to correct for small sample size) for model comparisons and present ΔAIC, where highly supported models have ΔAIC ≤2 compared to other models (Burnham et al., 2011). We also report AIC weights, which quantify the relative support for specific models and the terms within. Weights range from 0 to 1. We then performed model averaging of these candidate models for each morphological trait. Only conditional model averages are reported because shelterin protein gene expression and telomere length were each a priori included in only one candidate model. Because different Indiana nestlings were used for telomere and shelterin protein analyses, Indiana nestlings were not included in this analysis.

3 | RESULTS

Shelterin protein gene expression was significantly related to the latitude^2 term, such that nestlings from mid-range sites expressed nearly double the shelterin protein mRNA relative to more northern and southern sites (Figure 2b). PC1 gene expression was unrelated to sex, exact age, ageing method or brood size (Table 2). In contrast with shelterin protein gene expression, relative telomere length (Figure 2b) and proxies of growth (Figure 3) were not significantly related to any latitude terms, though these traits still showed marked intraspecific variation (see Tables 2 and 3 for full details). Telomere length was not significantly correlated with gene expression of any shelterin protein ($-0.1 < R < -0.02$, Figure S1).

TABLE 2 Linear mixed effects models assessing the relationship between latitude and covariates on relative telomere length and shelterin protein gene expression (PC1).

	Shelterin protein gene expression, $n = 168$			
	***β* estimate ± SE**	**df**	***F*-value**	***p*-value**
Intercept (known, female)[a]	105.67 ± 22.65			
Latitude	−5.71 ± 1.21	1, 8	0.39	0.55
Latitude^2	0.08 ± 0.02	1, 8	23.96	**0.001**
Nestling age	0.16 ± 0.15	1, 152	0.94	0.33
Ageing method (est.)	−0.18 ± 0.23	1, 152	0.27	0.61
Sex (male)	−0.16 ± 0.21	1, 152	0.53	0.47
Brood size	0.13 ± 0.09	1, 152	2.43	0.12
$R^2_m = 0.15$; $R^2_c = 0.15$				
	Relative telomere length, $n = 261$			
	***β* estimate ± SE**	**df**	***F*-value**	***p*-value**
Intercept (known, female)[a]	7.37 ± 6.04			
Latitude	−0.33 ± 0.31	1, 61	0.13	0.72
Latitude^2	0.004 ± 0.004	1, 61	1.28	0.26
Nestling age	−0.10 ± 0.07	1, 181	2.00	0.16
Ageing method (est.)	0.04 ± 0.06	1, 181	0.74	0.39
Sex (male)	−0.02 ± 0.05	1, 181	0.21	0.65
Brood size	0.02 ± 0.02	1, 181	0.71	0.40
$R^2_m = 0.02$; $R^2_c = 0.13$				

Note: All models included population and qPCR plate as random effects. Reference (intercept) levels for categorical variables are specified in parentheses. Marginal (R^2_m) and conditional (R^2_c) *R*-squared values represent the proportion of total variance explained by fixed, or fixed and random effects, respectively. Significant effects ($p \leq 0.05$) are bolded.

[a]Reference levels.

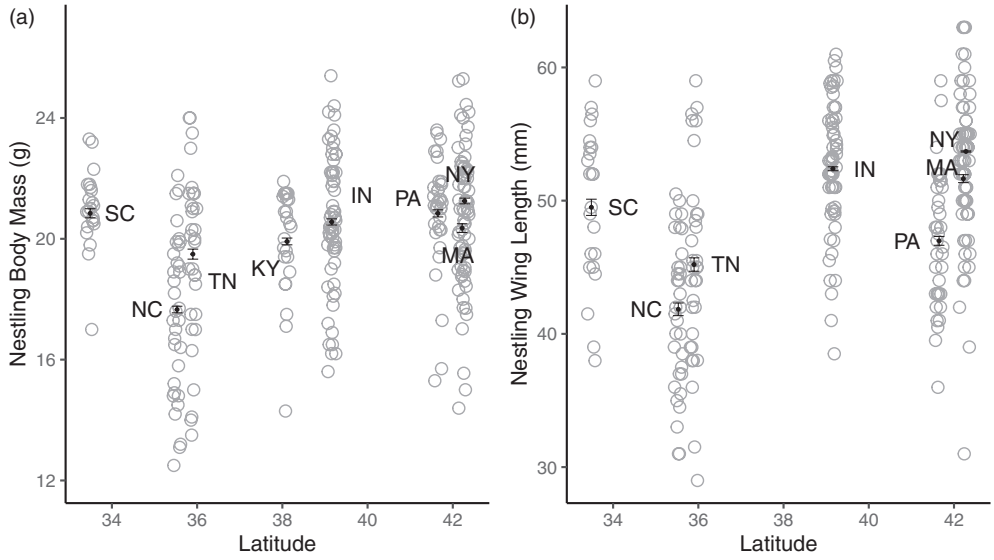

FIGURE 3 Morphological variation in age-matched nestlings across a latitudinal gradient in the eastern US: (a) body mass and (b) wing length. Points represent model outputs for average ± SE values per population, and grey circles are individual data points. Shaded areas show 95% confidence intervals.

TABLE 3 Linear mixed effects models assessing the relationship between latitude and proxies of nestling growth.

	Body mass (g), $n = 279$			
	β estimate ± SE	**df**	***F*-value**	***p*-value**
Intercept (known, female)[a]	109.91 ± 84.33			
Latitude	−5.21 ± 4.42	1, 5	1.51	0.27
Latitude^2	0.07 ± 0.06	1, 5.	0.98	0.37
Nestling age	0.67 ± 0.39	1, 267	2.29	0.13
Ageing method (est.)	−0.19 ± 0.46	1, 267	0.81	0.37
Sex (male)	0.24 ± 0.26	1, 267	0.60	0.44
Brood size	−0.63 ± 0.11	1, 267	31.09	**<0.0001**
$R^2_m = 0.16$; $R^2_c = 0.35$				
	Wing length (mm), $n = 255$			
	β estimate ± SE	**df**	***F*-value**	***p*-value**
Intercept (known, female)[a]	198.35 ± 224.33			
Latitude	−11.55 ± 11.77	1, 4	4.48	0.10
Latitude^2	0.16 ± 0.15	1, 4	1.13	0.35
Nestling age	5.50 ± 0.99	1, 244	22.45	**<0.0001**
Ageing method (est.)	−4.43 ± 1.18	1, 244	15.05	**0.0001**
Sex (male)	−0.14 ± 0.67	1, 244	0.07	0.80
Brood size	−0.32 ± 0.29	1, 244	1.18	0.28
$R^2_m = 0.26$; $R^2_c = 0.41$				

Note: All models included state as a random effect. Reference (intercept) levels for categorical variables are specified in parentheses. Marginal (R^2_m) and conditional (R^2_c) *R*-squared values represent the proportion of total variance explained by fixed, or fixed and random effects, respectively. Significant effects ($p \leq 0.05$) are bolded.

[a]Reference levels.

PC1 gene expression outperformed telomere length in predicting nestling growth (Table 4). The top-ranking models for mass and wing length contained shelterin protein gene expression, each with a model weight ≥0.75, showing strong evidence that nestlings with lower shelterin protein gene expression were heavier and had longer wings at 12 days old (Figure 4a). We found weaker evidence for a relationship

TABLE 4 Akaike's information criteria for general linear mixed effects models predicting nestling mass and wing length with either telomere length or shelterin protein gene expression (PC1).

	k	logLik	ΔAIC	Akaike weight
Nestling body mass (g), $n = 150$				
Null + Shelterin PC1	10	−322.93	0	0.77
Null	9	−325.61	3.06	0.17
Null + Telomere length	10	−325.51	5.15	0.06
Nestling wing length (mm), $n = 150$				
Null + Shelterin PC1	10	−453.00	0	0.99
Null	9	−460.40	12.49	0.002
Null + Telomere length	10	−459.29	12.58	0.002

Note: The base (or 'null') model includes latitude, latitude^2, age at sampling, ageing method, sex and brood size, with population as a random effect.

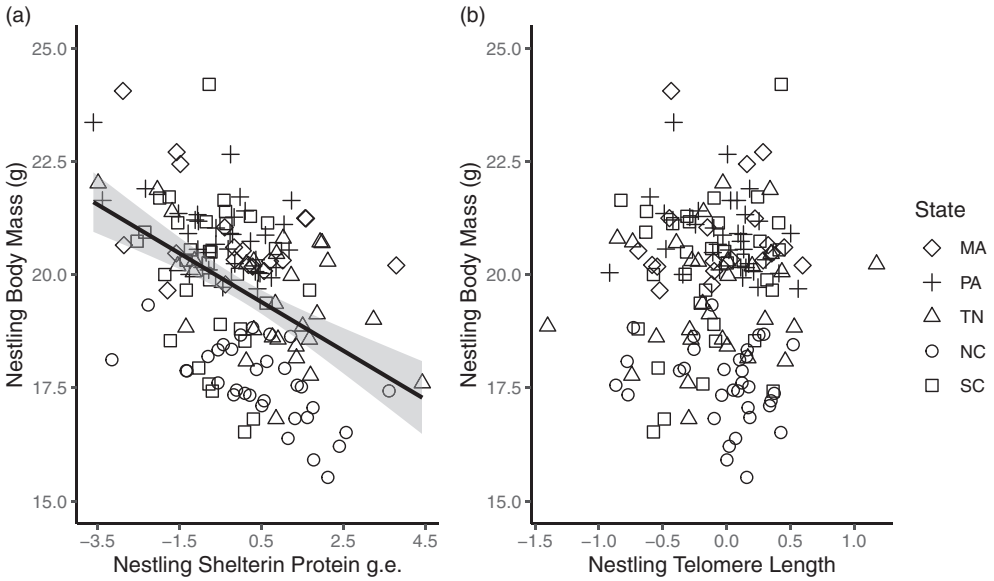

FIGURE 4 The relationship between body mass and (a) shelterin protein gene expression (PC1) or (b) relative telomere length (T/S ratio) among nestlings at 12 days old. One unit of PC1 equates to increases in gene expression of 50% for POT1 and 100% (or doubling) for TPP1, TRF2 and TRF2IP. Points represent individual nestlings. Shaded areas show 95% confidence intervals. Note that different Indiana nestlings were used for telomere and shelterin protein analyses, and so they could not be included here.

between telomere length and proxies of nestling growth (Table 4; Figure 4b), as all models containing telomere length and not shelterin protein gene expression had a ΔAIC ≥5.15, with model weights ≤0.06. Both 'null + shelterin' models also had more support than the null model alone, which had a ΔAIC ≥3.06. Furthermore, model averaging revealed that shelterin protein gene expression, but not telomere length, significantly predicted both nestling body mass and wing length (Table 5). Therefore, inclusion of shelterin protein gene expression in statistical models improved latitude- and age-based predictions of nestling growth and critically, outperformed models that included telomere length.

4 | DISCUSSION

Telomeres have been connected to environmental and intraspecific variation in life history (Burraco et al., 2020; Karkkainen et al., 2022; Kirby et al., 2017; Tricola et al., 2018; Whittemore et al., 2019), and we hypothesized that shelterin proteins may be key underlying mediators. We used tree swallows, which vary across populations in a number of environmental and life history traits like body size and growth (Ardia, 2005, 2006; Dunn et al., 2000; McCarty, 2001), a result replicated here using nearly 10° of latitude and updated to include the expanding southern range edge. Across these populations, we found significant non-linear latitudinal patterns in shelterin protein gene expression. Specifically, nestlings from mid-latitude sites expressed on average nearly double the shelterin mRNA compared to more northern and southern sites, and individual variation was even more marked (up to 256×). Because the pleiotropic effects of shelterin proteins are likely related to telomere dynamics and may affect physiology, we expected to also find intraspecific variation in telomere length and body size measured at the end of the growth period, the latter of which is an established proxy of growth and

TABLE 5 Conditional model-averaged coefficients for models predicting nestling body mass and wing length with either shelterin protein gene expression or telomere length.

Body mass (g) ($n = 150$)	β estimate ± SE	*F*-value	*p*-value
Intercept (known, female)[a]	188.33 ± 122.96	1.52	0.13
Shelterin protein gene expression	−0.35 ± 0.12	2.78	**0.005**
Telomere length	0.03 ± 0.44	0.07	0.94
Latitude	−9.62 ± 6.50	1.47	0.14
Latitude^2	0.13 ± 0.09	1.50	0.13
Nestling age	1.03 ± 0.22	4.60	**<0.0001**
Ageing method (est.)	0.09 ± 0.45	0.20	0.84
Brood size	−0.62 ± 0.14	4.30	**<0.0001**
Wing length (mm) ($n = 150$)	**β estimate ± SE**	***F*-value**	***p*-value**
Intercept (known, female)[a]	280.12 ± 186.56	1.49	0.14
Shelterin protein gene expression	−1.25 ± 0.31	3.97	**<0.0001**
Telomere length	0.39 ± 1.14	0.34	0.74
Latitude	−15.99 ± 9.88	1.60	0.11
Latitude^2	0.21 ± 0.13	1.62	0.10
Nestling age	5.68 ± 0.55	10.16	**<0.0001**
Ageing method (est.)	−3.62 ± 1.08	3.32	**0.0009**
Brood size	−0.15 ± 0.36	0.43	0.67

Note: Population was included as a random effect in all models. Significant effects ($p \leq 0.05$) are bolded.
[a]Reference levels.

predictor of future fecundity and survival (Haywood & Perrins, 1992; McCarty, 2001). While we did not find a relationship between telomere length and nestling morphology, we did find that shelterin protein mRNA abundance better predicted intraspecific variation in nestling size than did telomere length, altogether encouraging continued research on shelterin proteins in life history.

We predicted that shelterin protein gene expression would co-vary with latitude, and we found lower shelterin mRNA abundance at the northern and southern ends of our sampling range. Quadratic relationships with latitude are not uncommon (Karkkainen et al., 2022; Lappalainen et al., 2008) and may be driven by factors that act differentially with latitude. For example, shelterin proteins respond to food limitation (Rouan et al., 2021; Wolf et al., 2022), a major driver of population dynamics at more northern latitudes (MacArthur, 1984). Other factors may dominate in the south. For example, the southernmost population in this study (South Carolina) lies at the edge of an ongoing southward range expansion (Siefferman et al., 2023; Wright et al., 2019). Expanding populations, including those of the tree swallow, often exhibit unique sets of phenotypes like boldness or aggression (Siefferman et al., 2023), both energetically costly behaviours that may affect shelterin protein abundance (sensu Ludlow et al., 2013). Regardless of its cause, population averages differ by as much as twofold (a doubling) in their baseline gene expression. Among-individual variation within populations was even more marked, up to ~2.5 to 8 log2-fold, which translates to 6 to 256-fold variation in gene expression among individuals. While shelterin mRNA abundance has already been linked to stress resiliency and survival in adults and nestlings of this species, respectively (Wolf et al., 2022), we cannot yet determine the functional outcomes of this variation. At present, we have no evidence that this natural intraspecific variation compromises telomere functionality. As speculated below, latitudinal variation in shelterin levels may lead to differential regulation of physiology (e.g. metabolism and immune function) and the expression of life history traits across populations.

Telomere length, on the other hand, did not vary with latitude in 12-day-old nestlings. Our result is among others in asking how the environment drives spatial patterns of telomere length (reviewed in Burraco et al., 2021), for example long telomeres are found in low-latitude adult black bears (*Ursus americanus*) but *mid*-latitude nestling and adult pied flycatchers (*Ficedula hypoleuca*; Karkkainen et al., 2022; Kirby et al., 2017). Latitudinal differences may be masked by variation in telomere length *within* populations that results from variability in natal conditions and population-specific factors. Within-population variability may also shrink after the first year of life, at which point only 10%–20% of nestlings remain alive (Winkler et al., 2020). If so, latitudinal patterning may emerge among adults, though compensatory shifts in telomere regulation may equalize length across the range. However, strong directional selection on telomere regulation or length may be unlikely if it promotes non-functional telomeres or cancers. Continued population and longitudinal analyses are key to disentangling these alternatives.

That shelterin protein gene expression did not co-vary with telomere length may not be intuitive, but there are several reasons why this might be the case. The only major evidence of shelterin-telomere covariation comes from research on cancer (Fujii et al., 2008; Hu et al., 2010), a diseased state in which trait variation may far exceed that of putatively healthy wild animals. Shelterin-telomere covariation may be masked, first, by other telomere regulators like glucocorticoids (Angelier et al., 2018) and antioxidants (Badas et al., 2015). Second, shelterin abundance may be more temporally and environmentally plastic than telomere length (Belmaker et al., 2019; Chik et al., 2022), which may produce covariation only under specific conditions or windows of time. In addition, focusing sampling on median-massed, 12-day-old nestlings may limit variation in telomeric traits and, thereby, the probability of detecting relationships. Shelterin abundance may also be more strongly correlated with telomere length in tissues with greater telomerase activity than nucleated red blood cells (e.g. gonads; Haussmann et al., 2007). These findings underscore the need for a closer look at the dynamics of shelterin proteins and telomere length, and the relative role each plays in tracking versus contributing to life history.

Biomolecular work has begun to establish potential links between shelterin proteins and physiological traits that have ecological relevance (Akincilar et al., 2021; de Lange, 2018; Ye et al., 2014). Consistent with this view, we found that shelterin protein gene expression co-varied with two key proxies of growth, namely, mass and wing length measured after a period of rapid postnatal growth. Furthermore, shelterin protein gene expression was a stronger predictor of morphology than telomere length and improved upon our basic latitudinal model, suggesting that shelterin proteins may contribute to intraspecific variation in life history traits. The fact that shelterin is highly expressed in the blood of adult tree swallows despite negligible telomerase activity in the same tissue (Bentz et al., 2019) suggests that shelterin may act via telomere-independent functions. For example, high shelterin levels (e.g. RAP1, TIN2) are associated with metabolic dysfunction in cell culture (Chen et al., 2012; Teo et al., 2010). High shelterin gene expression was found in our smallest nestlings, whose slow postnatal growth is a robust predictor of lifespan (McCarty, 2001). Similarly, Wolf et al. (2022) reported higher shelterin POT1 gene expression in adult birds with smaller body mass and a stronger weight-loss response to sickness. Experimental manipulation of a shelterin gene (TRF1) also affects metabolism (Augereau et al., 2021). Continued efforts to characterize shelterin proteins in nature are vital to testing shelterin's effects on traits that vary within species and are visible to natural selection.

5 | CONCLUSIONS

We hypothesized that shelterin proteins may contribute to diversity in life history because these proteins may functionally connect telomere dynamics to physiological outcomes and life history traits. This hypothesis differs from the prevailing idea that telomeres are passive correlates of life history traits and, to date, remains largely untested. However, our *among*-population analyses corroborate and extend a few recent and exciting *within*-population analyses of shelterin proteins (Rouan et al., 2021; Wolf et al., 2022). In doing so, we underscore the need for further study of the shelterin proteins in an ecological context (as discussed in Wolf & Shalev, 2023). By applying these ideas to variation within and among species, we will move closer to understanding the proximate mechanisms that generate patterns of diversity in nature.

AUTHOR CONTRIBUTIONS

Sarah E. Wolf and Kimberly A. Rosvall designed the project; Sarah E. Wolf, Kimberly A. Rosvall, Mary J. Woodruff, David A. Chang van Oordt, Ethan D. Clotfelter, D.A. Cristol, Elizabeth P. Derryberry, Stephen M. Ferguson and Conor C. Taff collected samples; Kimberly A. Rosvall, Ethan D. Clotfelter, Elizabeth P. Derryberry, Mark T. Stanback, Maren N. Vitousek and David F. Westneat provided access to field sites; Sarah E. Wolf performed laboratory work and statistical analyses; Sarah E. Wolf and Kimberly A. Rosvall wrote the manuscript. All authors edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.w9ghx3fx6 (Wolf et al., 2024).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1: Primer sequences for gene expression analysis of shelterin proteins.

Table S2: Linear mixed effects models assessing the relationship between latitude and covariates on relative telomere length and shelterin protein gene expression (PC1), using qPCR plate ID as a fixed effect.

Figure S1: Pearson correlations between shelterin protein gene expression and relative telomere length in the blood of 12-day old tree swallows.

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