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Primers to highly conserved elements optimized for qPCR-based telomere length measurement in vertebrates

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Abstract

Telomere length dynamics are an established biomarker of health and ageing in animals. The study of telomeres in numerous species has been facilitated by methods to measure telomere length by real-time quantitative PCR (qPCR). In this method, telomere length is determined by quantifying the amount of telomeric DNA repeats in a sample and normalizing this to the total amount of genomic DNA. This normalization requires the development of genomic reference primers suitable for qPCR, which remains challenging in nonmodel organism with genomes that have not been sequenced. Here we report reference primers that can be used in qPCR to measure telomere lengths in any vertebrate species. We designed primer pairs to amplify genetic elements that are highly conserved between evolutionarily distant taxa and tested them in species that span the vertebrate tree of life. We report five primer pairs that meet the specificity and reproducibility standards of qPCR. In addition, we demonstrate an approach to choose the best primers for a given species by testing the primers on multiple individuals within a species and then applying an established computational tool. These reference primers can facilitate qPCR-based telomere length measurements in any vertebrate species of ecological or economic interest.

1 | INTRODUCTION

The measurement of telomere lengths is an important approach used to study the health and ageing of organisms. Telomeres are structures at the end of Eukaryotic chromosomes that consist of proteins bound to repetitive DNA sequences. Telomeres protect the ends of linear chromosomes and provide several important cellular functions (Gomes, Shay, & Wright, 2010). The length of telomere DNA shortens at each cell division, and telomere shortening can eventually lead to cellular senescence, which affects tissue function, organismal health and lifespan (McLennan et al., 2016; O'Sullivan & Karlseder, 2010; Wilbourn et al., 2018). Telomere

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lengths are considered an indicator of phenotypic quality (Bauch, Becker, & Verhulst, 2013; Saino et al., 2012) and telomere length dynamics have been shown to have predictive power of the future success of organisms. For example, telomere shortening has been shown to predict both lifespan and reproductive success (Eastwood et al., 2019; Heidinger et al., 2012), which are proxies for organismal fitness. Short telomere lengths have even been suggested to precede extinction events (Dupoué et al., 2017). In addition, telomere shortening has been shown to be accelerated by various forms of stress (Barnes, Fouquerel, & Opresko, 2019; Bauch, Boonekamp, Korsten, Mulder, & Verhulst, 2019; Tricola et al., 2018) and telomere lengths have been used to evaluate environmental quality (Martens & Nawrot, 2016; McLennan et al., 2016; Meillère, Brischoux, Ribout, & Angelier, 2015). The ability to monitor the well-being of organisms and to predict their future success has important applications in both wild and captive environments. In addition, understanding telomere dynamics across the vertebrate tree of life could also lead to a better understanding of the mechanisms and evolution of ageing in general.

Despite the motivation for measuring telomere lengths, research remains limited to relatively few species, with the majority of studies done in avian species (Bauch, Becker, & Verhulst, 2014; Beaulieu, Reichert, Le Maho, Ancel, & Criscuolo, 2011; Quirici, Guerrero, Krause, Wingfield, & Vásquez, 2016). Methodological challenges are one reason for the limited use of telomere length measurements. A widely used method is to measure relative telomere length by real-time quantitative PCR (qPCR). This method requires only common laboratory equipment and techniques, can be done in relatively high-throughput and is robust despite low sample quantity and quality (Lin, Smith, Esteves, & Drury, 2019; Martin-ruiz et al., 2015von). Measurement of telomere length by qPCR requires primers that amplify telomere repeats, where the concentration of telomeric DNA in a sample, determined by qPCR, is proportional to telomere length. The method also requires reference primers that amplify a nontelomeric region of the genome of interest to normalize for the total amount of genomic DNA in the sample (Cawthon, 2002). The repeating DNA sequence of telomeres (TTAGGG) is identical in all vertebrates, so the established telomere-specific primer pairs should work in any vertebrate species (Meyne, Ratliff, & Moyzis, 1989). In contrast, the genome-specific reference primers optimized in one species may not work in other species because of unknown genetic differences, often requiring the design and optimization of new primers for each new species that is to be studied (Horn, Robertson, & Gemmell, 2010; Smith, Turbill, & Penn, 2011). Reference primer development is especially challenging in nonmodel organisms with genomes that have not been sequenced. Despite the wide potential application of measuring telomere lengths by qPCR, reference primer design may limit the adoption of this assay in newly analysed species.

Here we report PCR primers that can be used as genomic reference primers for qPCR-based telomere length measurements in any vertebrate species. To design a qPCR assay that would work broadly in vertebrates, we designed primers to amplify ultraconserved elements (UCEs) which are genetic elements that are highly conserved between evolutionarily distant taxa (Asghar, Hasselquist, & Bensch, 2011; Bejerano, 2004; McCormack et al., 2012). We note that a UCE primer pair was previously developed by others and has been shown to be effective in telomere length assays in avian species (Asghar et al., 2011). We identified primer pairs that amplify five different UCEs and also meet qPCR requirements for specificity and efficiency (Bustin et al., 2009) (Figure 1a). We designed these primers to match the annealing temperature of the established telomere primers so that they can be used in the same PCR plate if desired. We found that all five primers efficiently and reproducibly amplify genomic DNA in every vertebrate sample that we tested. In addition, the targeted UCEs do not vary in copy number in the species we investigated. As a proof of concept, we used the primers to verify a significant decrease in telomere length with age in American kestrels, a species for which telomere lengths had not previously been measured. The reported qPCR primers enable the expansion of telomere length studies to new vertebrate systems for basic and applied research questions.

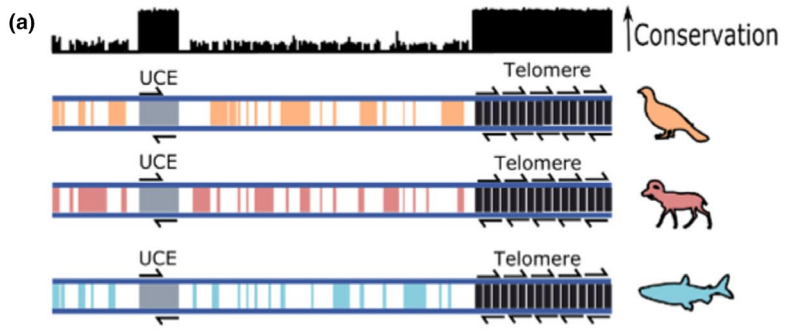
2 | MATERIAL AND METHODS

2.1 | Genomic DNA extraction

DNA was extracted from blood or tissue samples using the Zymo Quick-DNA Microprep Plus Kit (#D4074) according to the manufacturer's protocols for the sample type. Blood samples from northern goshawk (*Accipiter gentilis*) and American kestrel (*Falco sparverius*) were stored in Queen's lysis buffer (0.01 m Tris, 0.01 m NaCl, 0.01 m EDTA, and 1% n-lauroylsarcosine, pH 7.5) prior to extraction. Human DNA was from a buccal swab. DNA from a red-eared slider (*Trachemys scripta elegans*) was extracted from a shell fragment. DNA from sea squirt was extracted from whole organisms. All other DNA samples were extracted from muscle or liver tissue. All animals were treated in accordance with Boise State University animal care and use policies where applicable. DNA purity was assessed via the 260/280-nm absorbance ratio (NanoDrop). DNA was quantified by absorbance at 260 nm and the concentration was normalized to 2 ng/μl in 10 mm Tris-HCl pH 8.5 and 0.1 mm EDTA.

2.2 | Quantitative PCR

qPCR was performed using a Roche LC96. For each UCE primer pair, efficiencies were determined using a serial dilution of mouse DNA samples, performed in triplicate. The standard curve was prepared by seven serial dilutions (1:5). All qPCRs were carried out in 20-μl volumes containing ~8 ng DNA for unknown samples (or 4 μl of varying concentrations of DNA for serial dilutions), 10 μl of 2× Biotium Fast Plus EvaGreen qPCR Master Mix, 10 pmol each of forward and reverse primers (500 nm final primer concentration) and water up to 20 μl. For both UCE and telomere amplification, the



UCE	Efficiency	R^2
UCE.359	97%	0.99
UCE.28	95%	0.99
UCE.64	101%	0.99
UCE.239	101%	0.99
UCE.176	102%	0.99

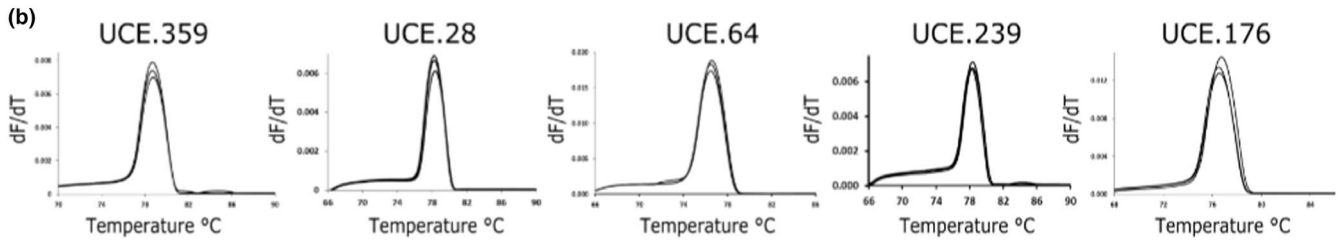


FIGURE 1 Ultraconserved element (UCE) primer performance in mouse. (a) Conceptual diagram of the vertebrate telomere assay design. Black bars represent the level of conservation of genomic regions determined by the alignment of multiple genomes such as a bird, a mammal and a fish. Telomeric repeats are conserved in all chordates and are amplified by primers specific to this sequence. The same reference primers can be used in all species because they amplify UCEs that have high conservation between distant taxa. (b) Melt curves of the amplification product of the qPCR of genomic mouse DNA using the best primer pair for each UCE. (c) Efficiencies and R^2 values of the best primer pair identified for five different UCEs, named by their access number in the UCE database

two-step thermal cycling profile was 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 55°C for 30 s, with signal acquisition at the end of the 55°C step and melt curves generated by increasing temperatures from 72 to 95°C, in 0.5°C steps, with a 30-s dwell period per step at the end of the thermal cycling. Amplification of kestrel samples were carried out under identical conditions, using the same thermal cycling. Reactions with previously reported primers for 36B4 in mice contained 250 nm final concentration of forward and reverse primers with all other reagents as described above. PCRs with the 36B4 primers were thermal cycled for 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s with a final extension for 5 min at 72°C and signal acquisition at the end of the 72°C step and melt curves generated by increasing temperatures from 72 to 95°C, in 0.5°C steps, with a 30-s dwell period per step at the end of the thermal cycling. PCRs for GAPDH (Forward 5′-CTAAGCGTGTTATCATCT-3′, Reverse 5′-ACTTGTCATACTTGTCAT-3′) were thermal cycled at 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 55°C for 30 s, with signal acquisition at the end of the 55°C step.

The Cq values determined for technical replicates were averaged and any sample with a quantification cycle (Cq) standard deviation of greater than 0.5 between triplicates or with Cq values outside of the standard curve were repeated. Concentrations (ng) of telomere repeats and UCEs were determined using an external standard curve approach and Roche LightCycler software (release 4.0) for absolute quantification auto-analysis with the second derivative maximum method (proprietary). The efficiencies of each primer pair were determined from the slope of the standard curves using Roche LightCycler software where $E = 10^{[-1/\text{slope}]}$. Optimal efficiency (100%) is defined as a slope of 3.32. The correlation coefficients (R^2) were determined from the replicates of the dilution series.

2.3 | Data analysis

For the 21 species in which we tested our primers, we constructed a phylogenetic tree based on the tree-of-life and its evolutionary timescale. We loaded the 21 species into TimeTree (timetree.org) and generated a phylogenetic tree that showed the 21 extant species and ancestral progenitors. Primer pairs were assessed for their effect on stability values computationally (geNorm) as previously described (Vandesompele et al., 2002) in four of the 21 vertebrates. The same genomic DNA samples from multiple individuals were amplified with three to five UCE primer pairs, and the Cq values were used to sequentially eliminate the least stable primer pair. The two most stable UCE primer pairs were then used to generate DNA concentrations (ng) from a standard curve and these concentrations were graphed on a scatter plot with Pearson correlations and trend lines reported. Genomic DNA from the organism of interest which had the highest concentration, measured by UV absorbance, was used for the standard curve. To calculate relative telomere lengths in adult and nestling kestrels, the telomere concentration (T) was divided by the average concentration of two UCE genes (UCE_{ave}) to yield $T/\text{UCE}_{\text{ave}}$.

3 | RESULTS

3.1 | Assay development

We set out to develop primer pairs to several UCEs to enable cross-validation. We chose several UCEs from UCbase 2.0 and designed multiple primer pairs for each using a primer design tool (OligoArchitect, Sigma-Aldrich). During design, we limited the product length to 250 bp and designed primers to have a T_m of 60°C to match the T_m of established telomere-specific primers (Roos et al., 2008). We ranked the primer pairs computationally (Beacon Designer) and experimentally tested the top four pairs for each of the UCEs by amplifying mouse DNA in a qPCR. Using melt-curve analysis, we identified primer pairs for five of the UCEs that produced a single-peaked melt curve (Figure 1b). We determined the amplification efficiency for each of these five UCE primer pairs by amplifying seven serial dilutions of the mouse DNA in triplicate (Figure 1c). A primer pair for all five UCEs was found that have qPCR efficiencies within the best-practice range of 90%–110% and correlations (R^2) of the Cq values for replicates ≥0.980 (Kuang, Yan, Genders, Granata, & Bishop, 2018) (Figure 1c). The sequences of the chosen UCE primer pairs is reported in Table 1.

3.2 | Validation across the vertebrate tree of life

We next tested our primers across the vertebrate tree of life (Figure 2a). We collected various tissue types from 19 species for DNA extraction. This generally was isolated from a single individual unless the DNA was of insufficient concentration to complete testing. To test our primers as broadly as possible, we also included a sea squirt, which is amendable to telomere length measurements because basal chordates have the same telomeric repeat sequences as vertebrates. We performed qPCR on each sample in triplicate, with all five primer pairs and evaluated the melt-curves. We found that all five primer pairs amplified DNA from every species with a single melt peak. These results indicate that all five primer pairs can amplify the ultraconserved regions in all vertebrates that we tested.

An additional requirement for reference genes used for normalizing telomere length is that they must not vary in copy number among individuals in the population. For example, a duplication of a reference gene in one individual would appear as a halving of their telomere lengths relative to a nonduplicated individual. The amplification of closely related homologue variants could also introduce normalization differences that would have the same effect as copy number variation. The challenge of identifying reference primers with minimal variation between individuals for telomere length measurements is similar to choosing primer pairs for normalizing real-time PCR data for gene expression. We therefore evaluated our primer pairs using an algorithm, geNorm, designed for this purpose (Vandesompele et al., 2002). The geNorm algorithm evaluates the cumulative variation among multiple primer pairs and iteratively eliminates primer pairs that contribute the most variation until final primer pairs are chosen. In this approach, stability values are a measurement of the pairwise variation between primers and therefore lower stability values are preferred. Two or more primer pairs are preferred over a single primer pair to reduce random experimental variation (D'haene, Vandesompele, & Hellemans, 2010). Using the NORMQPCR package in R, which utilizes the geNorm algorithm, we identified the best combination of UCE primer pairs from DNA extracted from 20 mice (*Mus musculus*), 17 rock ptarmigan (*Lagopus muta*) and 20 northern goshawks (*Accipiter gentilis*), representing both sexes and a range of ages. Samples were amplified by qPCR with each of the five reference primer sets using the same DNA concentration (8 ng) for each reaction. We found that the use of two primer pairs for normalization leads to very low stability values in each of the four species tested (Figure 2b). Next, we extracted DNA from another mammal (bighorn sheep, *Ovis canadensis*) and tested the top three primer pairs which had been identified in mouse. Using NORMQPCR we identified the two primer pairs with the lowest stability values (Figure 2b). The genomic DNA concentration quantified by these two primer pairs showed high correlation (Pearson),

TABLE 1 Ultraconserved element and telomere primer sequences

Name	Sequence
tel 1b	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
tel 2b	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
UCE.359-F	ATCTGAGACTTGTGACAT
UCE.359-R	GTGTTAATTGGTAATGACTATT
UCE.28-F	AAATACCACCCAACAGTTT
UCE.28-R	AAGCCCTATACAGATGGAT
UCE.64-F	GAGTCTCCAATATCATCAGAAGC
UCE.64-R	ACACATGCCACGATCAATG
UCE.239-F	TCAGATGTTCAGCCTATT
UCE.239-R	AATACCATGTTAATTATCCTCAA
UCE.176-F	TTTCTACAGTTCTGATTTAGTTGA
UCE.176-R	TGTTCCCTGTCGCATTAG

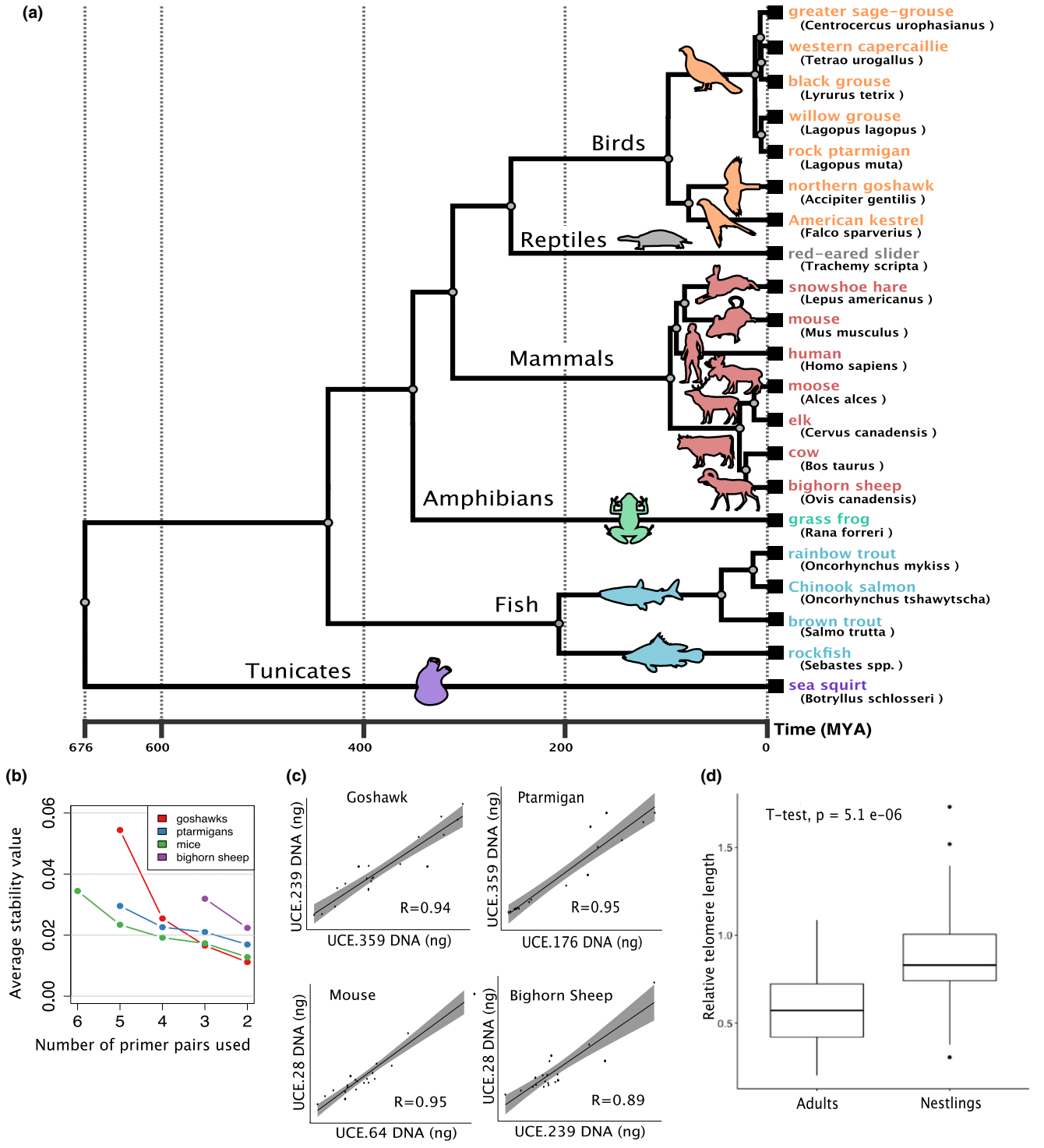


FIGURE 2 Validation of UCE reference primers across the vertebrate tree of life. (a) Phylogenetic tree of organisms in which reference primers were validated (timetree.org). (b) Average stability values (pairwise variation) generated with geNorm from the utilization of six to two reference gene primers. (c) Pearson correlation between the genomic DNA concentrations (ng) of the two UCE primer pairs that resulted in the lowest average stability values (geNorm) in each of four organisms. (d) Relative telomere length for adult and nestling American kestrels using UCE primers

suggesting that neither pair exhibits copy number polymorphisms within the individuals tested (Figure 2c; Figure S1). We also tested primer efficiency in these four animals and found that all primers tested fell within the recommended range of 90%–110% (Table S1). We also calculated intra-assay variation as a measurement of the average coefficient of variation between replicates and found that

it is between 0.26% and 2.17%, demonstrating strong repeatability (Table S2). It is beyond the scope of this work to examine stability and efficiencies in each of the 21 vertebrates in which we tested for UCE primer amplification, but we recommend that primer efficiency, melt curve analysis and stability be examined prior to adoption of the UCE reference primers.

We next set out to determine if the UCE-based assay yields expected relative telomere length measurements in a species where telomere lengths have not been previously measured. We extracted DNA from the blood of 15 adult and 29 nestling American kestrels (*Falco sparverius*). We amplified each DNA sample separately with telomere-specific primers, UCE.28 and UCE.239, which were chosen because of low stability values and high correlations. Relative telomere length was calculated as the ratio of the telomere amplification product to the average of the UCE amplification products. The results showed that adult American kestrels had a significantly shorter telomere length than nestlings (Figure 2d). We note that, prior to developing the UCE primers, we had attempted to utilize published GAPDH primers to normalize telomere lengths in the American kestrel. We found that both the published (Criscuolo et al., 2009) and extensively redesigned and optimized GAPDH primers exhibited unpredictable variation in the size of PCR products between individuals, making this commonly used reference gene unsuitable in this organism (Figure S2). These results suggest that our primers may even be an improvement for telomere assays in avian species.

3.3 | UCE primers are an improvement over previously reported telomere assay reference primers for mice

We next quantified 20 mouse samples with a reference primer pair previously reported in the literature that was designed for qPCR-based telomere length measurement in mice (Callicott & Womack, 2006). The previously reported primers target the acidic ribosomal phosphoprotein PO (36B4) gene and have been used in multiple publications (Callicott & Womack, 2006). We used the published thermal cycling profile of the 36B4 primers to determine the PCR efficiency and evaluate the melt peak. The 36B4 primers had a good efficiency (102%) but showed a broad, multipeaked melt-curve, indicating PCR artefacts or that nonspecific amplification had occurred (Figure S3). When we included the 36B4 primer pair in the NORMQPCR stability analysis for mouse, this primer pair was the second eliminated by the geNorm algorithm, indicating that our top UCE primer pairs are an improvement with respect to genomic reference stability values in mice. These results further suggest that our UCE reference primers may be useful even in organisms with established reference primers.

4 | DISCUSSION

Real-time qPCR-based telomere length assays provide a reliable and high-throughput method for studying telomeres. The primers reported here make this assay possible in a broad range of vertebrate species. However, care should be taken in developing and optimizing a qPCR assay in any new species because measurement error can occur within an assay, between assays and between multiple collections and extractions of DNA from tissue samples (Eastwood, Mulder, Verhulst, & Peters, 2018). Additionally, significant variation can occur from the qPCR reagents used (Morinha, Magalhães, & Blanco, 2020). The multiple reference primers reported here provide the opportunity to assess the variability introduced by the reference primers themselves. For the study of new species, it is advisable to first test all five UCE primer pairs on at least 10 individuals and perform a stability analysis to find the best two primer pairs (Vandesompele et al., 2002). Compared to a single reference primer pair, using the average quantity from two reference primer pairs for relative telomere length measurement has the advantage of averaging out other sources of qPCR noise (Kozera & Rapacz, 2013). While three or more reference primers could be used if warranted, this would require sufficient access to DNA and additional reagents. In the experiments reported here, we were able to identify primer pairs with low stability values in bighorn sheep by comparing the best three primer pairs identified from mouse DNA samples (UCE.28, UCE.64 and UCE.239). This suggests that these three primers could be a good starting point for any mammalian or ungulate species. However, the slightly lower correlation between the concentrations measured for the bighorn sheep samples, compared to the other organisms, suggests that a better primer pair might be found if all five primers were tested (Figure 2c). The bighorn sheep samples also had relatively low DNA concentration, which may have contributed to the lower correlation value. In addition to primer choice, the change in telomere lengths due to age and stress is species-specific, and the biological relevance of telomere lengths must be experimentally established in each organism.

While the UCE reference primers reported provide the opportunity to determine relative telomere length in multiple vertebrate species, direct comparison between species would require additional experimental validation. It is unclear that differences in absolute telomere length between species has much biological relevance, and recent studies have found that initial telomere length does not correlate with differences in lifespan between species (Nussey et al., 2014; Udroiu, 2020). The extent of interstitial telomeric repeats that exist between centromeres and telomeres in different species further complicates direct comparisons. Even in fully sequenced and annotated genomes, the number of interstitial repeats is hard to quantify because long repetitive regions are difficult to assess accurately. Interstitial telomere repeats have been measured in several bird species using a modified gel-based assay (Tricola et al., 2018). This previous report showed that the amount of interstitial telomere repeats can vary between species, and even between individuals of the same species (Foote, Vleck, & Vleck, 2013; Nussey et al., 2014). Despite the common use of qPCR for numerous telomere studies, this method is not able to distinguish interstitial telomeric repeats from terminal telomeres. This suggests that the qPCR signal from interstitial

telomeres could have a substantial influence on the variation in telomere lengths observed and prevent comparison between species. Interstitial telomere lengths are not known to change with age, so they should not interfere with the measurement of the rate of attrition determined when the same individual is measured at multiple time points. This may be why recent studies of telomeres in numerous species have shown significant correlation between telomere shortening rates and lifespans (Udroiu, 2020; Whittemore, Vera, Martínez-Nevado, Sanpera, & Blasco, 2019). Nevertheless, when establishing telomere length measurements in a new species, it is prudent to evaluate the contribution of interstitial telomere length contributions from the literature or using an alternative approach (Eastwood et al., 2018; Foote et al., 2013; Whittemore et al., 2019).

In addition to telomere length measurements, the UCE primers reported here may also be useful in other studies that require quantification of genomic DNA. For example, qPCR has been used to quantify blood parasite load in birds, which requires reference primers to the host's genome, and a UCE has been used for this purpose (Asghar et al., 2011). Copy number variation (CNV) within populations represents a large component of genetic diversity that affects normal and disease phenotypes (Freeman et al., 2006). The UCE primers presented here may also facilitate a more widespread evaluation of CNV in vertebrates, especially because the multiple UCE primers can be used to cross-validate the nonvariable nature of the reference primers in different genomes.

The ability to determine relative telomere length in any vertebrate species creates new opportunities in basic and applied research. For example, telomere length measurements can be used for monitoring the health and ageing of organisms. Telomere lengths have even been considered a proxy for fitness because telomere lengths and shortening rates have been shown to predict lifespan and reproductive success in some species (Bauch et al., 2013; Eastwood et al., 2019). Telomere lengths in indicator species in different environments may allow identification of important ecosystem disturbances, including those caused by humans. The ability to analyse multiple species simultaneously will allow the study of how telomere lengths change during long-term predator–prey cycles or other ecological interactions. The ability to study telomere length dynamics in diverse taxa will allow for a better understanding of ageing across the tree of life and from an evolutionary perspective (Bauch et al., 2013; Eastwood et al., 2019).

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CONFLICT OF INTEREST

None declared.

AUTHOR'S CONTRIBUTIONS

S.F.H. carried out the molecular laboratory work, participated in data analysis, participated in the design of the study and drafted the manuscript; E.P.H. carried out molecular laboratory work and participated in design of the study; J.D.B. participated in statistical analysis, data analysis and coding, S.J.B. participated in study design and data analysis; D.P.B. participated in study design, editing the manuscript and creating figures; K.R.C. carried out molecular laboratory work and participated in data analysis, J.S.F. critically revised the manuscript, collected field samples and provided funding; L.P.W., R.A.M. and O.K.N. critically revised the manuscript, and collected field samples, J.A.H. critically revised the manuscript, collected field samples, and provided funding; E.J.H. critically revised, conceived of the study, coordinated the study, helped draft the manuscript and provided funding. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

DATA AVAILABILITY STATEMENT

The data presented in this paper are available from the DRYAD database (https://doi.org/10.5061/dryad.9p8cz8wd9).

geNorm is open source and available in the R software package NORMQPCR (https://github.com/jimrperkins/NormqPCR).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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