

TELOMERE LENGTH AS A BIOMARKER FOR STRESS IN EASTERN BLUEBIRD
NESTLINGS (*SIALIA SIALIS*) REARED IN URBAN HABITATS

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requirements for the degree of Master of Science in Biology

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ABSTRACT

TELOMERE LENGTH AS A BIOMARKER FOR STRESS IN EASTERN BLUEBIRD NESTLINGS (*SIALIA SIALIS*) REARED IN URBAN HABITATS

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Eastern bluebirds (*Sialia sialis*) are often found in urban areas and have seemingly adapted to the changing conditions, however, an often-overlooked potential threat for urban dwellers is anthropogenic stress or human-induced environmental stressors. Anthropogenic stressors may initiate a physiological cascade which leads to oxidative damage, including telomere loss. The intricate relationship between stress (physiological and oxidative stress) and telomere length (TL) has led to the utilization of TL as an index for stress. I hypothesized that Eastern bluebird nestlings developing in more urbanized settings experience higher levels of stress. Moreover, I predicted that urban nestlings have shorter telomeres than rural nestlings. This is in line with previous studies that found an association between stress exposure and shortened telomeres in other urban-raised birds. In this study, I used telomere length (TL) as a biomarker for stress in bluebird nestlings reared in an urban-rural gradient. I measured the relative telomere length (TL) of 35 nestlings reared in urban (17) and rural (18) habitats located in Carroll County, GA using quantitative "real-time" PCR (qPCR). The technique involves the measurement of amplified target (T) telomere sequences relative to a reference single copy gene (S); GAPDH was chosen as the single copy gene due to its conserved nature in all passerines. I found that urban nestlings have shorter telomeres as demonstrated by their lower mean T/S ratios when compared to those of rural

nestlings. Therefore, the results of this study suggest that urban-reared Eastern bluebird nestlings are, in fact, stressed and incur a greater physiological cost than their rural counterparts. Future studies could explore the physiological processes behind the reduction of TL in Eastern bluebird nestlings by measuring the release of glucocorticoid stress hormones (e.g. CORT) in tandem with TL.

INTRODUCTION

Urbanization has accelerated in recent decades to the detriment of natural environments, yet many animals have colonized urban areas (Giraudeau & McGraw 2014; Jackson et al. 2011). Although extremely altered, urban areas offer new prospects such as enhanced feeding via scavenging or human feeding for certain adaptable species (Shanahan et al. 2014). For instance, European blackbirds (*Turdus merula*) regularly scavenge human garbage and use human structures for roosting and are consequently found in large numbers within human settlements (Partecke et al. 2006). However, urban colonizers face many potential threats. An often-overlooked potential threat to living in urban habitats is anthropogenic stress—stress caused by human activities (Angelier et al. 2013; Meillere et al. 2015). Even animals that are seemingly adapted to urban conditions may in fact be hindered by the constant exposure to anthropogenic environmental stressors, such as air pollutants, artificial lighting, and traffic noise. These are almost exclusively present in urban areas and pose unique challenges to urban living. Physiological and behavioral adaptations may equip some urban-dwelling animals with a greater capacity to withstand long exposure of anthropogenic stressors without incurring noticeable costs (Bize et al. 2009; Bonier et al. 2007; Partecke et al. 2006). However, urban colonizers may be more susceptible to anthropogenic stressors than previously thought and may, as a result, survive in urbanized habitats at a physiological cost; e.g. enhanced stress (Biard et al. 2017; Meillere et al. 2017).

Like any type of stressor, anthropogenic stressors may activate a physiological response in vertebrates via the hypothalamic-pituitary-adrenal (HPA) axis—a complex set of neuroendocrine feedback interactions responsible for the discharge of glucocorticoid hormones (Hausmann et al. 2012; Hausmann & Marchetto 2010) including corticosterone (CORT) which is the main

glucocorticoid stress hormone in birds (Astheimer et al. 1992). CORT and other glucocorticoid hormones are normally secreted at low levels but can spike in response to stress (Quirici et al. 2016). Over-secretion of glucocorticoid stress hormones can increase production of reactive oxygen species (ROS), which are damaging by-products of metabolism and can intensify oxidative damage (Epel et al. 2004; Haussmann et al. 2012). Moreover, glucocorticoids can encourage oxidative stress by incapacitating dietary or enzymatic antioxidants, thus hampering the capacity to regulate the concentration of ROS, also known as free radicals (Haussmann & Marchetto 2010). As ROS accumulate they begin to damage key biological molecules like DNA and proteins. However, ROS selectively target and cleave G-rich sequences primarily found in telomeric DNA (Oikawa & Kawanishi 1999), which makes telomeres particularly susceptible to oxidative damage and results in the shortening of telomeric ends (Haussmann et al. 2012). Telomeres are often thought of as crucial ‘caps’ which add stability to linear chromosomes and prevent damage to genomic DNA (gDNA) (Delany et al. 2003; Haussmann & Marchetto 2010). Vertebrate telomeres are non-coding, guanine-rich sequences that are arranged in tandem (TTAGGG) repeats and are located at chromosomal ends (Delany et al. 2003). Telomeres naturally shorten at every replication event during cell division—the “end-replication-problem” (Monaghan 2014). Once telomeric deterioration passes a certain size threshold, apoptosis (cell death) or replicative senescence normally follow; senescent cells remain in a non-replicative stage and do not contribute to tissue regeneration (Horn et al. 2010; Monaghan & Haussmann 2006). Premature cell death and replicative senescence are thought to be chief causes of aging and increased age-related mortality in organisms. Excessive telomere loss can predict remaining lifespan (Angelier et al. 2013; Barrett et al. 2013; Bize et al. 2009; Boonekamp et al. 2014; Cawthon et al. 2003; Hammers et al. 2015; Haussmann et al. 2005; Heidinger et al. 2012; Horn et al. 2010; Pauliny et al. 2006). However, this

is not always the case and is far from the full story, as stress (CORT and oxidative stress) and telomere length (TL) are intricately connected (Hausmann & Marchetto 2010; Quirici et al. 2016), which is why faster telomere erosion rates and smaller telomeres may be used as indices for oxidative stress, and in turn indicate heightened physiological stress (Hausmann et al. 2012). In other words, TL can be used as a biomarker for stress. In this study, I attempt to determine whether same-age urban Eastern bluebird nestlings (*Sialia sialis*) are more stressed than their rural counterparts by comparing their TL.

It is becoming clear that telomere loss likely reflects both the proliferative history of cells and the accumulation of oxidative damage following persistent stress (Collado et al. 2007), and the considerable variation in TL and the amount of telomere change among same-age individuals (Hall et al. 2004) and even cell cultures (von Zglinicki 2002) mostly reflects different telomere attrition rates resulting from oxidative stress (von Zglinicki 2002). Far from solely playing the role of ‘mitotic clock’, telomeres (telomere dynamics and TL) instead likely act as gauges of an organism’s current and future capacity to fight off stress and the telomeric degradation that follows (Hausmann et al. 2012; Monaghan & Hausmann 2006). In addition, telomere attrition transpires at a faster rate in young animals because growth and development are still taking place (Hall et al. 2004), which is further magnified in fast-growing animals with correspondingly faster cellular turnover rates than slower-growing animals (Dantzer & Fletcher 2015; Tissier et al. 2014). Passerine birds are altricial, meaning they hatch premature and vulnerable, and must grow exceptionally fast to survive (Boonekamp et al. 2014). As such, they are particularly exceptionally susceptible to significant replicative and oxidative telomere loss early in life (Beaulieu et al. 2011; Boonekamp et al. 2014; Caprioli et al. 2013; Foote et al. 2010; Herborn et al. 2014; Meillere et al. 2015; Mitzutani et al. 2013). Therefore, natal environmental conditions are crucial in influencing

TL of birds during their nestling phase and into adulthood (Herborn et al. 2014; Meillere et al. 2015) and may subsequently decrease their survivability into adulthood (Heidinger et al. 2012). Urban areas are often suboptimal habitats and are inundated with anthropogenic stressors; e.g. traffic noise, artificial lighting, and air pollutants (Biard et al. 2017; Isaksson 2010; Meillere et al. 2015; Salmon et al. 2016). Nestlings growing up in urban areas must contend with potentially demanding environmental conditions, and the physiological costs that may come with growing up in them. Previous studies have found an association between stress exposure and telomere attrition in certain species of urban-raised wild birds (Meillere et al. 2015; Salmon et al. 2016). Nevertheless, there is a relative dearth of studies investigating the stress and its effects on animals living in urban centers (Barrett et al. 2013; Bonier 2012; Isakson 2010; Nussey et al. 2014). Fewer still have studied stress by measuring TL of same-age nestlings (Biard et al. 2017; Meillere et al. 2015; Quirici et al. 2016; Salmon et al. 2016) and, to my knowledge, those that have done so have not focused on Eastern bluebird nestlings. In this study, I test the hypothesis that Eastern bluebird nestlings reared in urban habitats are more prone to stress and thus will have shorter telomeres than eastern bluebird nestlings reared in rural environments. Telomere length (TL) is used as a biomarker for stress, where shorter TL signifies higher levels of stress (Barrett et al. 2013; Heborn et al. 2014; Meillere et al. 2015; Nussey et al. 2014; Salmon et al. 2016).

Working with Eastern bluebirds presents an exceptional opportunity to assess the potential physiological costs of living in urban environments. As obligate secondary cavity-nesters, bluebirds depend upon natural nest sites previously excavated by primary cavity nesters (Pinkowski 1977; Sauer & Droege 1990) or on man-made nest boxes for breeding. Bluebird nestlings reared in nest boxes are therefore easily accessible, and their age can be easily determined. Also, bluebirds have seemingly adapted to human-altered environments, their success

is partially being due to their ability to colonize territories in or around urban centers, where most of the nest boxes have been placed (Jackson et al. 2011). Here they forage and breed, and inadvertently expose their young to an overabundance of pesticides, air pollutants, poorer food choices, and other anthropogenic stressors (Jackson et al. 2011). For all their adaptive capacity, urban bluebirds are likely not exempt from sublethal physiological costs (e.g. CORT overload, oxidative stress, and telomere loss) that follow prolonged exposure to these anthropogenic stressors; nestlings more than adults. Like other passerines bluebird nestlings go through a period of fast growth and hence have fast cellular turnover rates (Beaulieu et al. 2011; Herborn et al. 2014; Mitzutani et al. 2013). Bluebird nestlings may, in turn, incur more stress in urban areas than in more sparsely populated areas.

METHODS

Study Site & Sample Collection

Data was collected from March through August 2013 from a total of 125 nest boxes which were established in spring of 2011—all located in Carroll County, GA (33.5642° N, 85.0649° W). Nest boxes were made from cedar and mounted on a 1.5m aluminum pole with a metal conical predator guard attached to the pole just beneath the box. Nest boxes were placed along an urban to rural range, all evenly separated from each other; approximately 100 meters (m) apart. Rural sites were characterized by pastures or agricultural fields, mostly devoid of humans, whereas urban sites were characterized by areas of high human traffic such as within the city limits of Carrollton, GA, or within the University of West Georgia campus (Graham 2014). Of the 125 nest boxes, 62 were in urban areas, while 63 were in rural sites. Newly active nests were checked every 3 days to establish first egg date for each nest and hatching date, and every 3 days. Small blood samples (50-100 μ l) were collected from nestlings via brachial venipuncture during the start of the breeding season between 2011 and 2013. All sampled nestlings were 10-13 days old (Graham 2014). It was crucial to control for age since this was not a longitudinal study—nestlings were not followed and resampled over an extended period. Nestlings from the same box are likely to have similarities in TL due to genetic similarities and nesting environmental similarities. To control for possible confounds of genetic and maternal effects, I randomly selected one nestling per nest box and later included in the experimental qPCR plates, which acted as a representative per nest box. And henceforth 35 nest boxes (17 urban/18 rural) were ultimately used in this study for the following reasons: not all nest boxes were occupied by Eastern bluebirds, bluebird eggs in other nest boxes did not hatch/nest abandoned, hatched nestlings did not survive to 10-13 days due to nest predation

or other factors, or sampled nestlings could not be used. Avian blood is an ideal source of DNA template for telomere studies because, in addition to being very mitotically mobile, avian red blood cells (erythrocytes) are nucleated and numerous (Barrett et al. 2013; Nussey et al. 2014). Using avian erythrocytes thus allows for high throughput DNA extraction for minimal available blood when compared to mammalian blood (Hall et al. 2004; Nussey et al. 2014). Genomic DNA (gDNA) was later extracted from all blood samples using a ‘Promega’ gDNA kit and stored at approximately -17°C. The DNA concentration and purity of all samples were determined with a NanoDrop 1000 Spectrophotometer (Thermo-Scientific). Purity was evaluated using 260/280 and 260/230 ratios.

qPCR Assay and Plate Setup

TL of Eastern bluebird nestlings was measured using a quantitative “real time” PCR (qPCR) method established for use in birds (Angelier et al. 2013; Cawthon 2002; Criscuolo et al. 2009), with minor adjustments. Although not perfect, qPCR is ideal for field studies because it allows researchers to screen large sample sizes with less effort and within a relatively short period of time (Nussey et al. 2014). The technique can produce high-output data from small DNA samples, which an attractive feature for field workers studying animals that yield miniscule blood volumes upon extraction (Atema et al. 2013; Criscuolo et al. 2009; Nussey et al. 2014). The technique involves the measurement of amplified target (T) telomere sequences relative to a reference single copy gene or scg (S) (Cawthon 2002; Criscuolo et al. 2009). Due to the repeating nature of telomeric DNA, telomere primers are relatively non-specific and commence amplification at many sites within the telomeric regions of chromosomes. The resulting amplified product is proportional to the relative quantity of telomeric DNA at each chromosomal end (Cawthon 2002). Conversely, there is only one site where single copy gene primers anneal or bind

to in the entire genome (Aydinonat et al. 2014; Cawthon 2002; Criscuolo et al. 2009). In other words, the specificity of the scg primers and the resulting amplification can be used to normalize for the telomeric amplification; generate a relative TL measurement. I chose GAPDH as the scg for my study because it has been standardized for passerine birds and has been successfully used to measure RTL by qPCR in passerine birds (Angelier et al. 2013; Atema et al. 2013; Beaulieu et al. 2011; Bize et al. 2009; Criscuolo et al. 2009). GAPDH primers were designed from American redstart (*Setophaga ruticilla*) (RS) DNA, as originally specified by Angelier et al. (2013). Telomere primers (Tel1b/Tel2b) were first designed by Cawthon (2002) and later adapted by Epel et al. (2004) to work in all vertebrates (Table 2.1; Appendix A). Because they are conserved across all vertebrate taxa (Barrett et al. 2013; Delany et al. 2003) RS GAPDH (F/R) and telomere (Tel1b/Tel2b) primers were diluted to 400nM and 900nM, respectively, following a protocol modification of Angelier et al. (2013). These primer concentrations were optimized to reduce the unwanted formation of primer dimers while enhancing the amplification of target telomere sequences and the GAPDH gene, respectively, in this assay (Quirici et al. 2016).

Each reaction totaled 25µl and contained the following reagents: 12.5µl Power SYBR® Green Master Mix (Applied Biosystems), 1µl of each forward and reverse primer solution, Millipore water (MPW), and template (Table 2.2 & 2.3). Template and MPW volumes varied per reaction, as these volumes were adjusted accordingly per reaction to ensure that all nestling sample reactions contained a total of 10ng of DNA; each sample contained different DNA concentrations. I used the Quirici et al. telomere and GAPDH cycling protocols, with a few modifications (Fig. 2.2-2.5; Appendix B). My telomere cycling conditions were as follows: 10min at 95°C followed by 30 cycles of 1min at 95°C, 1min at 54°C, and 1min at 60°C. GAPDH cycling conditions consisted of 10min at 95°C followed by 40 cycles of 1min at 95°C, 1min at 62°C, and 1min at 60°C

(Quirici et al. 2016). Melt curve/dissociation analyses were performed after amplification to confirm specificity of primer sets (Fig. 6.1-6.4; Appendix C). Melt curve cycling parameters followed those suggested for plates using SYBR Green reagents by the ABI High Resolution Melting starter guide. For GAPDH plates, these consisted of 40 cycles of 10sec at 95°C, 1min at 60°C, 15sec at 95°C, and 15sec at 60°C. The telomeres plates followed the same melt curve cycling parameters albeit set for 30 cycles. Both telomere (T) plates formed a single peak at approximately 78°C. GAPDH (S) plates formed a single peak at approximately 85°C, confirming specificity of both primer sets. I later ran an agarose gel containing the reaction products post melt curve analysis to further confirm specificity of primer sets (Fig. 7; Appendix C).

I randomly selected an adult sample and used it as the standard sample for all 96-well plates to produce a reliable standard curve which in turn is used to test the efficiency of each PCR reaction (Quirici et al. 2016). This standard was run on a 1:2 dilution series with 4 dilution points (80ng, 40ng, 20ng, 10ng of DNA) and in triplicates in all plates. The serial dilutions produced a reference curve used to control for the amplification within each plate to test amplification efficiencies within each reaction (accepted range $100\pm 15\%$, Quirici et al. 2016). Moreover, I prepared a no template control (NTC) reaction for each of the plates to control for reaction contamination, and to differentiate unintentional amplification products such as primer dimers from the intended products (Bustin et al. 2009). A reference nestling sample was run in triplicates on every plate for additional among-plate comparative capabilities (Quirici et al. 2016). All other (target) nestling samples were run in duplicates (Quirici et al. 2016) and fit into each of the telomere and GAPDH plates. Reactions were vortexed using an VWR Fixed Speed Vortex Mixer and centrifuged at 1,500 rpm for 5 minutes using an Allegra X-14 Centrifuge (Beckman Coulter) prior to amplification.

Telomere and GAPDH plates were run in an Applied Biosystems 7500 Real Time PCR instrument (Thermo-Scientific), utilizing the standard curve experiment option.

Relative Telomere Measurement

C_t values were calculated for all samples by the ABI 7500 qPCR instrument's analysis program as it gathered the data. The C_t value represents the number of PCR cycles required for a starting template to produce enough product to cross a critical threshold, or C_t , above baseline fluorescence and commence exponential amplification (Cawthon 2002; Criscuolo et al. 2009). Before this point, baseline fluorescence dominates the amplification plot and renders the detection and measurement of significant fluorescent signal impossible. Respective C_t values from Tel (T) and GAPDH (S) plates were later entered into the following formula: telomere length = $2^{\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t \text{ Tel} - C_t \text{ GAPDH})_{\text{reference}} - (C_t \text{ Tel} - C_t \text{ GAPDH})_{\text{target}}$ (Heidinger et al. 2012; Quirici et al. 2016), which is a variation of the $2^{(-\Delta C_t)}$ formula used by Cawthon (2002) and Criscuolo et al. (2009). Both formulas calculate the relative T/S ratio of telomere and GAPDH, or the length differences of telomeric DNA amplicon relative to the constant amplification of GAPDH product, per sample and thus yield the approximate quantity of telomere sequence within each individual nestling (Cawthon 2002; Criscuolo et al. 2009). qPCR amplifications were performed in two qPCR amplifications twice in two separate plates containing different primers (two telomere and two GAPDH plates) and the duplicate T/S ratios of each nestling were, in turn, used to calculate the mean T/S values of each nestling (Quirici et al. 2016). Furthermore, I determined the inter-plate variation by cross-analyzing the C_t values of individual samples with their inter-plate duplicate and calculated the coefficient of variation (%CV). The mean inter-plate %CV was 2.28% and 1.64% for the telomere and GAPDH C_t values, respectively.

Statistical Analysis

Data were reported as mean T/S ratios \pm standard deviation (SD). I conducted an unpaired, two-tailed t-test to determine whether the mean T/S ratios of urban and rural nestling samples were significantly different. All statistical analyses were performed using Microsoft Excel 2016.

RESULTS

I tested the hypothesis that nestlings reared in urban habitats are more stressed than those in rural habitats, and how this affects telomere length (TL). Mean TL of same age (10-13 days old) nestlings were significantly shorter in rural habitats (Fig. 1). The mean T/S ratio for urban nestlings ($\bar{x} \pm SD = 0.9034 \pm 0.4633$) significantly smaller than that of rural nestlings ($\bar{x} \pm SD = 1.393 \pm 0.5910$); $t_{32} = -2.6893$, $p = .011578$. The same pattern was observed in the two, plate replicates.

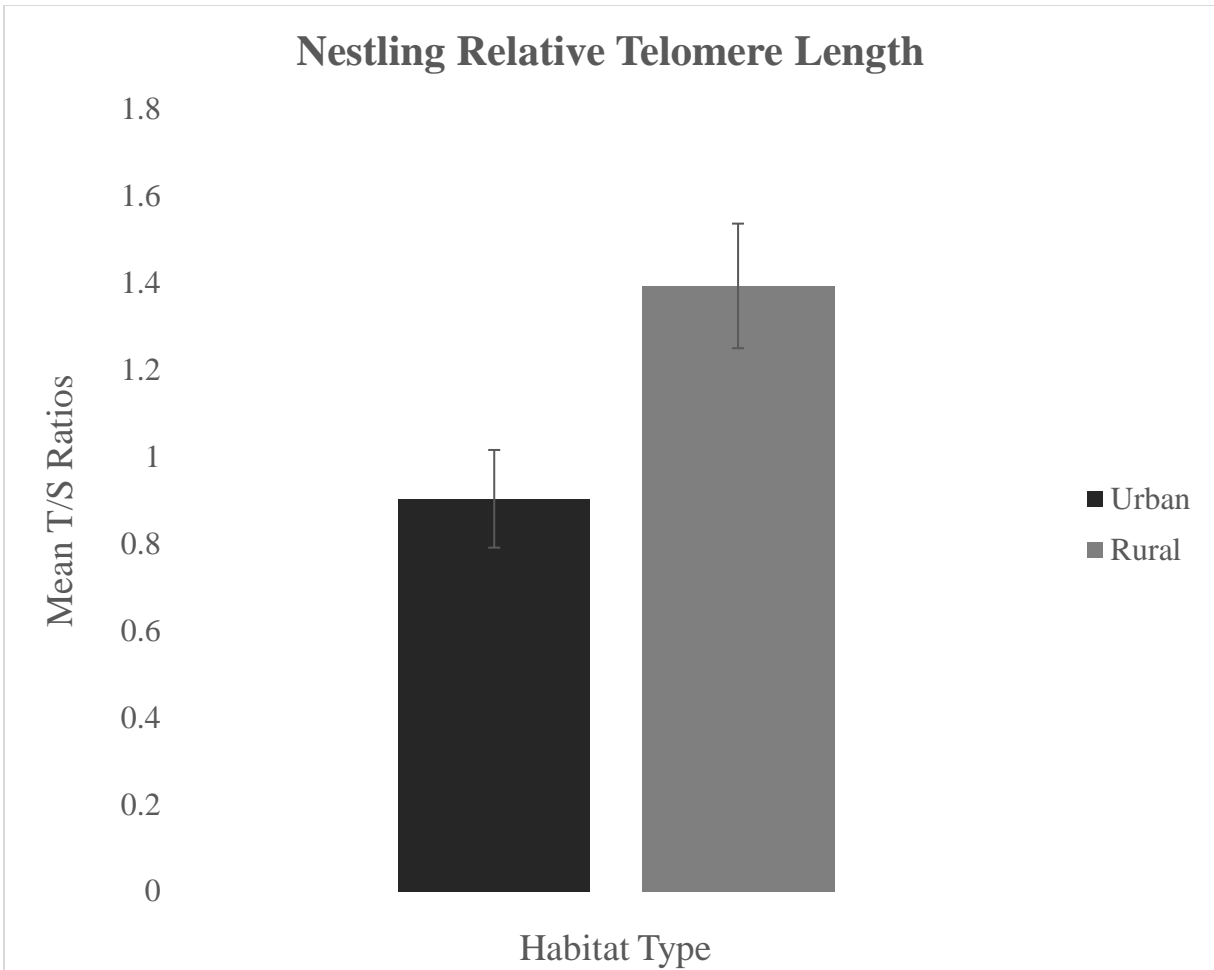


Figure 1. Telomere length (TL) of nestlings. Mean telomere length (mean T/S ratio \pm SD) of 10-13-day-old nestlings in urban and rural habitats, respectively. Urban habitat, N = 17. Rural habitat, N = 17. Post hoc t-tests showed a significant difference between urban ($\bar{x} \pm SD = 0.9034 \pm 0.4633$) and rural ($\bar{x} \pm SD = 1.393 \pm 0.5910$) T/S ratios; $t(32) = -2.6893$, $p = .011578$.

DISCUSSION

The objective of this study was to determine whether same-age urban Eastern bluebird nestlings are more stressed than their rural counterparts by comparing their TL. Differences in TL among nestlings of the same age (10-13-day-old) were expected to indicate different levels of stress: shorter TL signify higher levels of stress (Barrett et al. 2013; Heborn et al. 2014; Meillere et al. 2015; Nussey et al. 2014; Salmon et al. 2016). I measured the relative telomere length (RTL) of Eastern bluebird nestlings using a quantitative “real time” PCR (qPCR) method established for use in birds (Angelier et al. 2013; Cawthon 2002; Criscuolo et al. 2009), with slight modifications following assay optimization. Following this experiment, I observed significantly shorter telomeres among urban-reared Eastern bluebird nestlings when compared to rural-reared nestlings (Fig. 1). The mean inter-plate coefficient of variation (CV) was within an acceptable range: 2.28% and 1.64% for telomere and GAPDH C_t values, correspondingly. As predicted and consistent with other studies (Meillere et al. 2015; Salmon et al. 2016), urban nestlings possess shorter telomeres than rural nestlings. Shortened telomeres are an indicator of oxidative stress and thus my results show, for the first time, that even a relatively brief exposure (just short of two weeks) to an urbanized environment shortens the telomeres of Eastern bluebird nestlings. It is likely that urban nestlings experience higher levels of stress following a physiological cascade that is initiated by anthropogenic stressors. Consequently, this study provides support for the hypothesis that urban-reared Eastern bluebird nestlings experience higher levels of stress than rural-reared nestlings and that anthropogenic stress may have long term consequences for urban colonizers.

Eastern bluebird adults that colonize urbanized areas are not only putting themselves at risk, but their offspring as well. Survival rate and life expectancy generally decrease with

decreasing TL and/or hastening telomere attrition (Bize et al. 2009; Hall et al. 2004; Heidinger et al. 2012; Salomons et al. 2009). For instance, telomere rate of loss predicts survival rates in Alpine swifts (*Apus melba*) (Bize et al. 2009). More significant to my study, however, exposure to stressors early in life and the associated telomere loss persist throughout life (Aydinonat et al. 2014; Price et al. 2013), as seen in African grey parrots (*Psittacus erithacus erithacus*). If socially-isolated since birth, these birds retain shorter telomeres than their counterparts into adulthood (Aydinonat et al. 2014). Per previous authors (Biard et al. 2017; Herborn et al. 2014; Meillere et al. 2015; Quirici et al. 2016; Salmon et al. 2016), I interpret shorter telomeres (smaller T/S ratios) as indicative of higher stress. Furthermore, the offspring of urban-colonizing bluebirds might retain significantly higher stress levels into adulthood than their rural counterparts. And this pattern may compound over successive generations of urban bluebirds, as in other birds (Hausmann & Heidinger 2015).

I am unable to identify the exact source of stress in Eastern bluebird nestlings. But, nonetheless, shorter telomeres in urban Eastern bluebirds are consistent with other studies on wild birds living in suboptimal/potentially stressful habitats. For instance, adult American redstarts (*Setophaga ruticilla*) wintering in low-quality habitats were shown to have telomeres that were shorter than those of individuals that wintered in high-quality habitat (Angelier et al. 2013). Similarly, Thorn-tailed rayadito nestlings (*Aphrastura spinicauda*) reared in lower-quality, low latitude habitats have shorter telomeres and higher CORT levels than nestlings reared in higher-quality, high latitude habitats (Quirici et al. 2016). Although urbanized areas offer some benefits for colonizers, such as lower predation rates, decreased competition, and higher quality resources (Graham 2014), they are also often considered suboptimal habitats by several other metrics. For example, they are inundated with anthropogenic noise (e.g. traffic noise, Meillere et al. 2015;

Salmon et al. 2016), lighting (Isakson 2007), and air pollutants (Biard et al. 2017). Noisy areas are known to shorten TL in house sparrow (Meillere et al. 2015) and great tit nestlings (Salmon et al. 2016). Air pollutants originating from vehicular exhaust are also implicated in the observed differences in TL between urban and rural populations of great tit nestlings (Salmon et al. 2016). Consequently, while there may be some

There is a strong association between stress and TL (Angelier et al. 2013; Aydinonat et al. 2014; Epel et al. 2004; Haussmann et al. 2012; Meillere et al. 2015; Quirici et al. 2016; Salmon et al. 2016; Tissier et al. 2014; von Zglinicki 2002). Although the underlying mechanisms responsible for shortened telomeres observed in this study can only be inferred, as only TL was measured, previous studies have shown that stress hormone release and oxidative agents are major contributors to telomere loss (Angelier et al. 2013; Haussmann & Marchetto 2010; Pauliny et al. 2006; Quirici et al. 2016). Past longitudinal studies have recorded how surges in the production of glucocorticoids and ROS following HPA activation (Haussmann & Heidinger 2015; Monaghan et al. 2009) promote telomere loss, senescence and can therefore be linked to organismal life expectancy (Hammers et al. 2015; Haussmann & Heidinger 2015). Recurrent and prolonged release of glucocorticoid stress hormones leads to increased production of reactive oxygen species (ROS), inactivation of antioxidants, and hastening of telomere attrition (Haussmann et al. 2012). Maternal provisioning of glucocorticoid stress hormones often increases oxidative stress and telomere loss in offspring (Haussmann & Marchetto 2010). For instance, in domestic chicken embryos (*Gallus domesticus*), prenatal exposure to CORT in ovum intensifies oxidative stress and telomere loss, and these side-effects persist postnatally (Haussmann et al. 2012). Similarly, captive-bred zebra finch adult females (*Taeniopygia guttata*) injected with CORT produce nestlings with decreased growth rates and faster rates of telomere attrition, and themselves have

faster rates of telomere attrition (Tissier et al. 2014). Moreover, many pollutants are pro-oxidants and can themselves directly contribute to oxidative damage, including but not limited to telomere attrition (Hou et al. 2012; Salmon et al. 2018). In other words, airborne pro-oxidant pollutants like PM and nitrous oxides directly attack biomolecules such as telomeres independent of stress hormone regulation. This has been found in humans (Grahame & Schlesinger 2012; Hou et al. 2012; Hoxha et al. 2009) and in wild birds (Salmon et al. 2018). As such, higher stress and physiological consequences may follow in animals that have colonized urbanized areas, as I found in urban bluebirds. However, there are some benefits to living in urban areas and certain urban colonizers may have found ways to exploit urban resources while withstanding human disturbance. For instance, in the same Eastern bluebird system I studied, Graham (2014) found that males in urban areas are more aggressive than males in rural areas and suggested that there is likely differential selection imposed upon urban males for increased boldness and aggression, where these behaviors are favored. Unfortunately, not only has the loss of telomeres associated with chronic stress and its associated consequences has largely been overlooked in wild (Nussey et al. 2014), urban-colonizing animals but cost-benefit analyses of living in urbanized areas have also not been considered.

Although there is increasing evidence for higher stress in wild animals living in urbanized areas through the measurement of TL or telomere dynamics (Meillere et al. 2015; Salmon et al. 2016), one study found no such impact of urbanization (Biard et al. 2017). This is seemingly contradictory with the findings of the other aforementioned studies, and my own. Nevertheless, Biard et al. (2017) did find a negative effect on other measures of stress. One possibility for this discrepancy is that the observed trend of telomere attrition is, to some extent, species specific (Biard et al. 2017). However, this is not a particularly consistent nor explanatory solution. Another

possibility for confounding results might derive from improper accounting of genetic and environmental similarities among nestlings of the same nest box. Siblings sharing the same nest box likely share inherited-TL and the same developmental conditions. In my study, I controlled for genetic and nesting site environmental conditions among siblings by randomly including only one nestling per nest box into qPCR experimental plates. Each selected sample henceforth acted as a nest box representative and enhanced the significance of TL measurements and the overall strength of this study, which is in line with previous studies which showed that TL is at least partially inherited either genetically (Horn et al. 2011) or epigenetically (through maternal effects; Haussmann et al. 2012; Tissier et al. 2014). However minor, when compared to other factors, disregarding inheritance, nesting site environmental conditions, or maternal effects might otherwise lead to misleading results and would likely require further statistical analysis to interpret.

Overall, future studies should measure the release of glucocorticoid stress hormones (e.g. CORT) in tandem with TL of Eastern bluebirds and other passerine species. Several studies have performed simultaneous analyses of TL, oxidative stress, and glucocorticoid production in other species (Angelier et al. 2013; Biard et al. 2017; Quirici et al. 2016), however, this has not been done in bluebirds. The concentration of CORT could be measured from freshly-extracted blood before DNA digestion and other preparatory steps involved in making DNA samples (Quirici et al. 2016). Moreover, other physical measures such as body weight and tarsus length could be included as body quality measures, which are widely associated with accrued stress (Angelier et al. 2013; Biard et al. 2017; Quirici et al. 2016). Sampling could be done at multiple stages during nestling development, making additional CORT and TL measurements after each new blood sampling to compare differences that occur between sampling. Additional CORT and TL measurements taken concurrently at different time intervals would allow for multiple snapshots of

incurred stress, and therefore a more comprehensive window into nestling stress. Furthermore, there have been some indications that the magnitude of telomere loss may depend not only on the size of individual urban centers, but also on the species being studied (Biard et al. 2017). Therefore, other urban dwelling species should also be studied in the aforementioned manner in multiple urban centers of different sizes to measure the degree to which they are stressed in each urban size category, if they are affected at all.

In summary, few studies have investigated stress in wild urban-dwelling birds, fewer still have used TL as a biomarker for stress, and, to my knowledge, no other study has focused on Eastern bluebirds. My findings, for the first time, provide evidence of stress in urban Eastern bluebird nestlings of the same age. These results emphasize the necessity to more thoroughly explore the physiological consequences that befall urban-dwelling animals, specifically animals that are more susceptible to the costs of physiological stress; i.e. fast-growing passerine nestlings (Beaulieu et al. 2011; Caprioli et al. 2013; Foote et al. 2010; Herborn et al. 2014; Meillere et al. 2015; Mitzutani et al. 2013). As urban sprawl increases, and ever more natural habitat is converted for human use, urban-dwelling wildlife must adapt or be extirpated (Bonier 2012; Jackson et al. 2011; Shanahan et al. 2014). Urban areas generally favor species that can exploit more diverse food sources, including human-derived food, and can use non-native vegetation or man-made structures (Biard et al. 2017). For instance, Eastern bluebirds are regularly found in golf courses and other urbanized areas, where they hunt and breed, despite these areas often containing copious amounts of pesticides and other potentially harmful anthropogenic stressors (Jackson et al. 2011). However, these urban adapters/exploiters are likely not exempt from experiencing the physiological consequences associated with exposure to pressures/stressors (e.g. traffic noise, air pollutants, artificial lighting) that are ubiquitous in urban areas but are generally absent elsewhere

(Bonier 2012; Shanahan et al. 2014). Only by understanding the impact of human-derived stressors on urban-dwelling animals like the Eastern bluebird can we be able to act accordingly and make our urban centers less harmful to wildlife and ourselves.

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APPENDIX A – Optimization

Thermocycling Apparatus

Optimization began with standard PCR runs performed using an Eppendorf Mastercycler personal PCR apparatus. Quantitative analyses were later performed with an ABI 7500 Real Time PCR System.

Standard PCR Assay Optimization

Assay optimization began with the Criscuolo et al. assay protocol. The protocol involved diluting single copy gene (scg) GAPDH primers (F/R) from 100 μ M to 200nM. These GAPDH primers were designed using BLAST sequence data derived from zebra finch (*Taeniopygia guttata*) DNA. Other reagent specifications, such as master mix (MM) and Millipore water (MPW) volumes, were specified only in terms of standard/assumed qPCR volumes calculated by the machine in use. As Criscuolo et al. only performed qPCR runs—their paper did not contain standard PCR optimization step. Therefore, preemptive optimization steps using standard PCR were deemed necessary before delving into the primary qPCR methods of this project. I used the above-mentioned zebra finch (ZF) GAPDH scg primers diluted to 200nM with four eastern bluebird (BB) adult samples (76, 20, 24, 25) of differing DNA concentrations as templates, along with a negative control. All sample wells contained 1 μ l of each of the ZF GAPDH primers, 12.5 μ l of the master mix, 7.5 μ l Millipore water (MPW), and 3 μ l of template. The negative control contained equivalent volumes of MPW to compensate for the lack of template. The reactions were then run using the Criscuolo et al. GAPDH cycling program: an initial boiling step at 95°C for 10min, followed by 40 cycles of 60°C and 95°C each for 1min. Gel electrophoresis was initially performed using the following specifications: 1% agarose gel (0.3g per 30mL TAE buffer), 20 μ l

of 1.0mg/mL EtBr, 5 μ l of a 50bp DNA ladder, 5 μ l of PCR product mixed with 2 μ l of a loading dye per well in the gel, run at 75V for 45min.

After several PCR and gel electrophoresis runs without successful amplification, some gel optimization factors were proposed: increase gel thickness (perhaps agarose % too low), duration of electrophoresis (reduction of run time), and volume of DNA ladder used in run (too much DNA ladder). Gel thickness was increased to 2% agarose gel (0.6g per 30mL TAE buffer), DNA ladder volume used in well reduced to 1.5 μ l, and electrophoresis run time reduced to 30min. Moreover, sample 76 (21.6ng/ μ l) was used as template due to its higher DNA concentration when compared with all other samples. This was deemed important because there was some suspicion over the quality of DNA template; whether the BB samples were still viable. These modifications, however, did not produce noticeable changes.

PCR product volume was increased from 5 μ l to 20 μ l along with 4 μ l loading dye per well. Moreover, 5 μ l of 0.625mg/mL Ethidium bromide (EtBr) was added to the agarose gel before solidification, and gel thickness was maintained at 2%, which allowed for the detection of clearly separated DNA ladder. However, sample amplification bands were still not visible. Having barred all other factors suspected, I thus suspected primer concentration as the main issue hindering effective production/detection of amplicons. I questioned whether the Criscuolo et al. primers/primer concentrations were indeed optimal for bluebirds, despite having worked well with DNA template from other passerine species. I decided to incorporate several changes that would allow for effective comparison of amplification with different GAPDH primers and their specificity to each species. The following assay contained American redstart (RS) GAPDH primers (Quirici et al. 2016) and ZF GAPDH primers. Primers were used in the following concentrations: ZF GAPDH 6 μ M, ZF GAPDH 200nM, RS GAPDH 6 μ M, and RS GAPDH 200nM. I used either

song sparrow (*Melospiza melodia*) (SSP) DNA or bluebird (BB) DNA as template in each reaction tube. Each template (SSP or BB) was combined with each respective primer concentration to compare the amplification efficiency side by side. Negative controls were produced for every primer concentration. Gel electrophoresis conditions were largely like previous runs with several exceptions. First, two gels were cast to accommodate all products, each with different EtBr volumes due to gel size differences—20 μ l EtBr for 30ml gel cast and 10 μ l EtBr for 20ml gel cast. Second, a total of 24 μ l was used in every individual well (20 μ l product plus 4 μ l loading dye). Bands were observed in all wells containing the 6 μ M GAPDH primers (ZF/RS) and with both SSP and BB DNA as template. Furthermore, wells containing 6 μ M ZF/RS GAPDH primers showed bands at the expected length of 50bp and 300bp, respectively. However, no apparent bands were seen in the remaining lanes: those with 200nM GAPDH primers and the negative controls. These findings suggested that primer concentration was indeed the primary issue preventing amplification. Contamination of template and/or reagents was also ruled out.

ZF and RS GAPDH primers were further diluted to 2.5 μ M and run with template from both species. All other specifications remained the same. Bands of expected sizes were plainly visible in all lanes: 50bp in those with the ZF GAPDH and approximately 300bp in those with RS GAPDH (Fig. 2). All sample reactions from this point were carried out exclusively with adult BB template. Sample 76 was chosen as template for all remaining standard PCR runs due to its DNA concentration (11.6ng/ μ l) and purity. Progressively diluted ZF GAPDH primer concentrations (2 μ M, 900nM, 400nM) were tested in the next run to further refine the PCR assay. All other specifications remained the same except for the agarose gel thickness, which was increased from 2% to 2.5% in the expectation of better imaging after gel electrophoresis. There was no detectable amplification in any of the samples, which suggested that the ZF GAPDH primer dilutions were

not optimal; at least for standard PCR and should not be included in the project's final assays. Further optimization with qPCR was required.

Similarly, RS GAPDH primers were diluted to 2 μ M, 900nM, and 400nM from stock concentrations (100 μ M). Clear amplification resulted in some bands, producing bands of approximately 300bp (expected amplicon size). Although, both wells containing 2 μ M primers (negative control and sample) exhibited primer dimer formation; a result that was absent in all other wells. The sample well containing 900nM primers produced the brightest band. When combined with the latest ZF GAPDH results, it was revealed that although amplification occurred in all wells some primer concentrations were more favorable than others; at least while using standard PCR. Furthermore, RS GAPDH primers produced amplicons at lower concentrations than ZF GAPDH primers. Further optimizing with the qPCR instrument was required, however, these findings pointed to RS GAPDH producing more detectable/reliable results using qPCR. This is due to the sensitivity of the instrument to fluorescent signal when compared with standard imaging methods (UV transillumination of agarose gels).

The next step involved optimizing telomere primer concentrations. The telomere primer pairs (Tel1b and Tel2b) were diluted to 4 μ M, 2 μ M, 900nM, and 400nM from the stock solution (100 μ M). Each of these diluted primer pairs were later inserted into two solution tubes represented as either negative control or sample tube. All other PCR conditions remained the same. Gel electrophoresis also remained the same, apart from an increase in run time from 20 min to 25 min. Smearing was observed in all wells containing template, as expected, though the clearest smearing was seen in the well containing telomere primers diluted to 400nM. More importantly, however, this gel demonstrated that effective amplification was maintained while decreasing or eliminating primer dimer formation as the primer concentration decreased—barely noticeable in the 900nM

well and having completely vanished in the 400nM well (Fig. 3). Thus, telomere amplification lacking significant primer dimer formation was observed using primers at concentrations of 400 to 900nM. This was consistent with the Quirici et al. qPCR protocol and was indicative of a final working telomere primer concentration in my assay. Nevertheless, qPCR optimization was still necessary.

One last standard PCR run was deemed necessary before transitioning to qPCR optimization trials. Another set of primer concentrations (those used by Quirici et al. for both telomere and GAPDH primers) were put to the test alongside the previously assayed Tel 400nM. Telomere and GAPDH primers were established with differing forward and reverse primer concentrations—400nM (forward primer) and 900nM (reverse primer), respectively. As expected, smearing was observed in both telomere sample wells, although more apparent smearing was seen in the well containing the Quirici et al. telomere primer concentration combination: Tel1b (400nM) and Tel2b (900nM) (Fig. 4). This was countered by no significant band in the GAPDH well using the same primer concentration scheme. Further optimization with the qPCR was thought necessary.

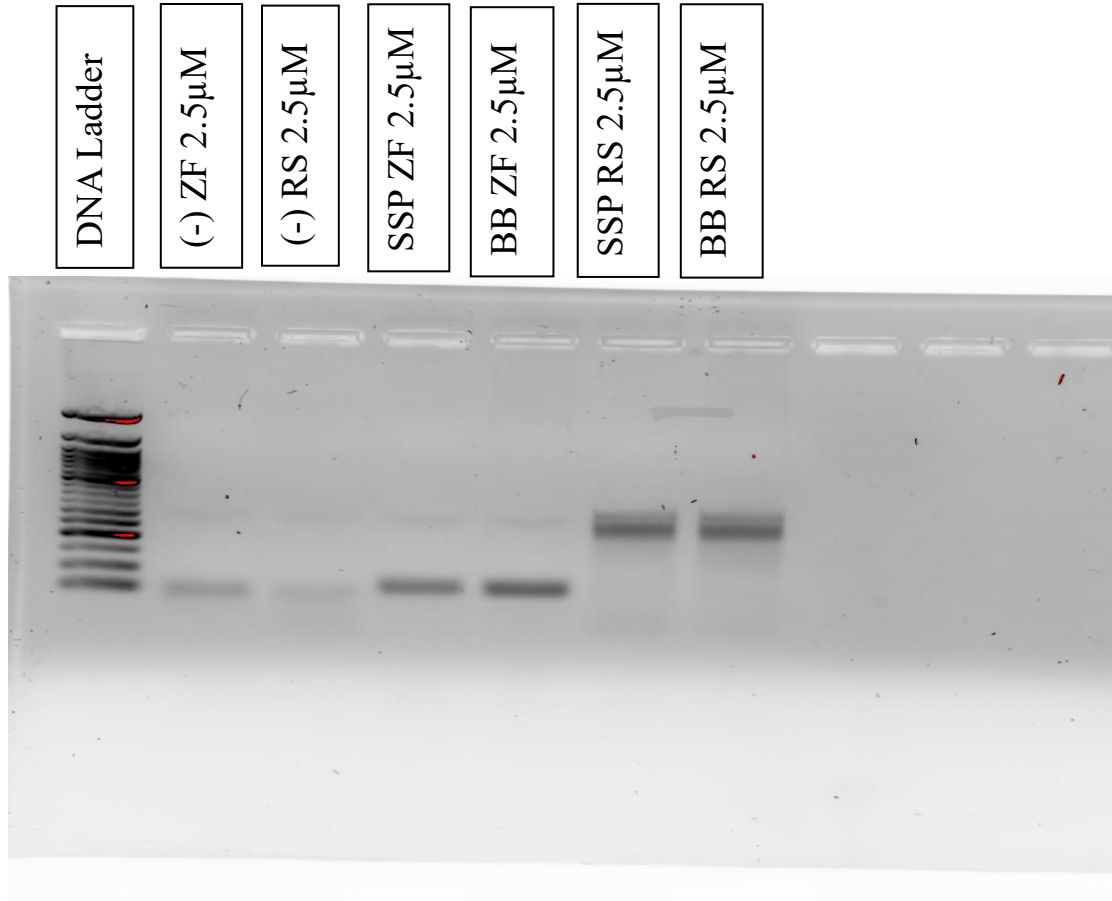


Figure 2. First optimization gel containing standard PCR products. Gel depicts expected ZF and RS GAPDH amplicons using song sparrow (SSP) and bluebird (BB) DNA, respectively. ZF GAPDH primers produced dsDNA amplicons approximately 50bp in length from both SSP and BB DNA whereas RS GAPDH primers produced amplicons that were approximately 300bp.

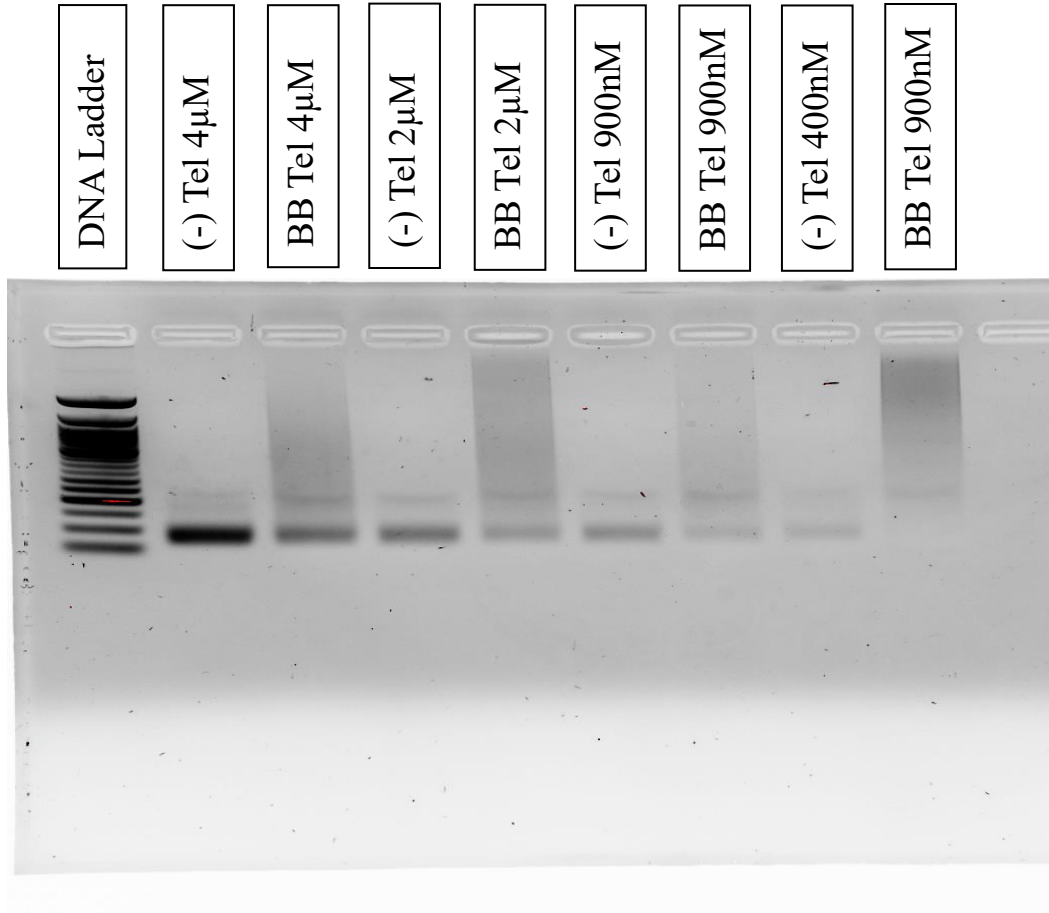


Figure 3. Second optimization gel containing standard PCR products. Gel depicts expected smearing in all reactions containing telomere primer sets and bluebird (BB) template DNA. Tel 900nM primer concentration confirmed as most favorable primer concentration due to generated smearing and apparent lack of primer dimer formation.

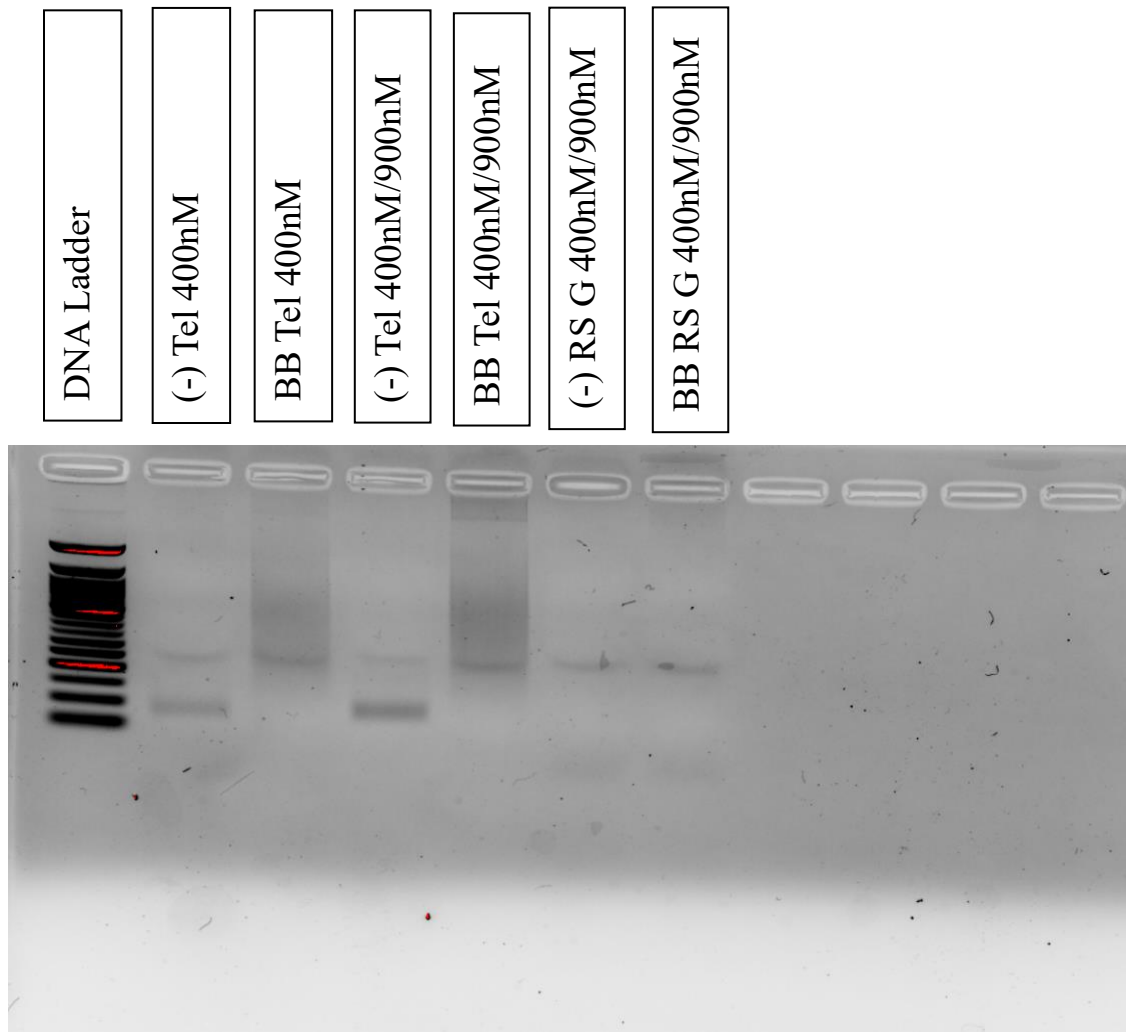


Figure 4. Third optimization gel containing standard PCR products. Gel depicts expected smearing in both reactions containing telomere primer sets and bluebird (BB) template DNA. Quirici et al. primer concentrations (Tel 400nM/900nM) produced most favorable results; easily visible smearing and no primer dimer formation. Quirici et al. GAPDH primer concentrations (RS GAPDH 400nM/900nM) did not work.

qPCR Assay Optimization

To start off the qPCR optimization trials, ZF GAPDH and RS GAPDH primers were tested for performance. Two concentrations (400nM and 200nM) were used for each GAPDH type (ZF/RS) but with the same forward and reverse primer concentrations, respectively; unlike the Quirici et al. arrangement. In total, four columns were arranged in the 96-well plate: ZF GAPDH (400nM), ZF GAPDH (200nM), RS GAPDH (400nM), and RS GAPDH (200nM). One no template controls (NTC) were produced for each primer category. The rest of the wells made up a 1:2 serial dilution of a standard sample (sample 76) developed to construct a standard curve. This serial dilution consisted of 4 dilution points (50ng, 25ng, 12.5ng, 6.25ng) repeated twice. These reactions were all run using a modification of the Quirici et al. GAPDH cycling protocol: 10min at 95°C followed by 40 cycles of 1min at 95°C, 1 min at 62°C, and 1min at 60°C. This modification was initially made because the ABI 7500 did not allow for the implementation of the official protocol; Quirici et al. step durations were too short. Nevertheless, these specifications produced favorable amplification curves in wells with the RS GAPDH primers set to 400nM. Furthermore, the R^2 value of these dilution curves indicated a good fit: 0.924. A subsequent gel electrophoresis run further confirmed the production of RS GAPDH amplicons in the wells with the primers set to 400nM (Fig. 5). Some amplification observed in wells containing 200nM RS GAPDH primers, but sharp curves were not seen. Wells containing ZF GAPDH, set to either concentration, did not produce significant results; either barely passed the fluorescence threshold or missed it entirely. ZF GAPDH were subsequently dropped from further optimization steps.

A couple of other experiments of similar composition were conducted using two additional adult bluebirds as template—samples 20 (8.5ng/μl) and 24 (10.1ng/μl), respectively. Serial dilutions (1:2) were used using the same specifications—starting template concentration, number

of dilution points, and number of replicates. Modifications were implemented as per the findings of the last run and included the following: ZF GAPDH primers were excluded, RS GAPDH primers were only used at a concentration of 400nM, and different time step durations in the Quirici et al. cycling protocol. Changes to the cycling time step were conducted to better emulate the original Quirici et al. cycling protocol, however, as previously mentioned, an exact recreation of the original cycling conditions was not possible. The ABI 7500 did not allow for the extremely short time durations of the original scheme: 20s at 95°C followed by 40 cycles of 3s at 95°C, 10s at 62°C, and 20s at 60°C (Quirici et al. 2016). The most similar imitation of the original went as follows: 5min at 95°C followed by 40 cycles of 15s at 95°C, 30s at 62°C, and 1min at 60°C. Surprisingly, this simulated cycling did not yield favorable results. In fact, although amplification curves were observed as in the previous standard curve experiment, the dilution products did not form a good fit on the standard curve line; $R^2=0.456$. This led to the exclusive implementation of the originally modified Quirici et al. GAPDH cycling protocol—95°C followed by 40 cycles of 1min at 95°C, 1 min at 62°C, and 1min at 60°C—in all future experiments amplifying GAPDH. The last two standard GAPDH runs implanted this optimized cycling regime to amplify with samples 20 and 24, correspondingly. This resulted in R^2 values of 0.936 and 0.923, respectively.

Several 1:2 serial dilutions were established using telomere primers and samples 20 and 24 as template. Sample 76 was at this point completely depleted as thus could not be used in further optimization tests. All the remaining standard curve experiments involved samples 20 and 24 set up with the same specifications as in earlier runs. Moreover, all reactions from this point were run with the Quirici et al. telomere cycling protocol. Each succeeding run, however, tested one of the following parameters: primer concentration, primer configuration, and cycling time intervals in the Quirici et al. telomere cycling protocol. The first run incorporated the closest imitation of the

original cycling protocol allowed by the ABI 7500 system: 5min at 95°C followed by 30 cycles of 15s at 95°C, 30s at 54°C, and 1min at 60°C. The run included sample 20 as template along with telomere primers set to three primer concentration arrangements—tel1b/tel2b (400nM), tel1b/tel2b (900nM), and tel1b(400nM)/tel2b(900nM). Despite all wells amplifying and producing clear curves, the standard curve results were less than acceptable ($R^2 = 0, 0.105, \text{ and } 0.622$, respectively). The tel1b(400nM)/tel2b(900nM) primer configuration was repeated since it produced the most favorable R^2 out of the set. The same specifications were preserved but only sample 24 was run as template; sample 20 was depleted. Surprisingly, this run's R^2 value was worse than was gathered in the preceding run (0.34). Suspecting the cycling time intervals as the causative factor producing these results, I settled on implementing the same time interval configuration as I had used in the GAPDH cycling. I superimposed these cycling time intervals onto the Quirici et al. telomere cycling temperatures—10min at 95°C followed by 30 cycles of 1min at 95°C, 1min at 54°C, and 1min at 60°C. Nevertheless, the following run with the tel1b(400nM)/tel2b(900nM) primer arrangement gave rise to subpar R^2 (0.728). Therefore, we concluded that this primer configuration was not working for my assay, and so we attempted another run using both telomere primers set to 900nM. This final run resulted in clear amplification curves along with a favorable R^2 (0.915), and thus pointed to this telomere assay as being optimized.

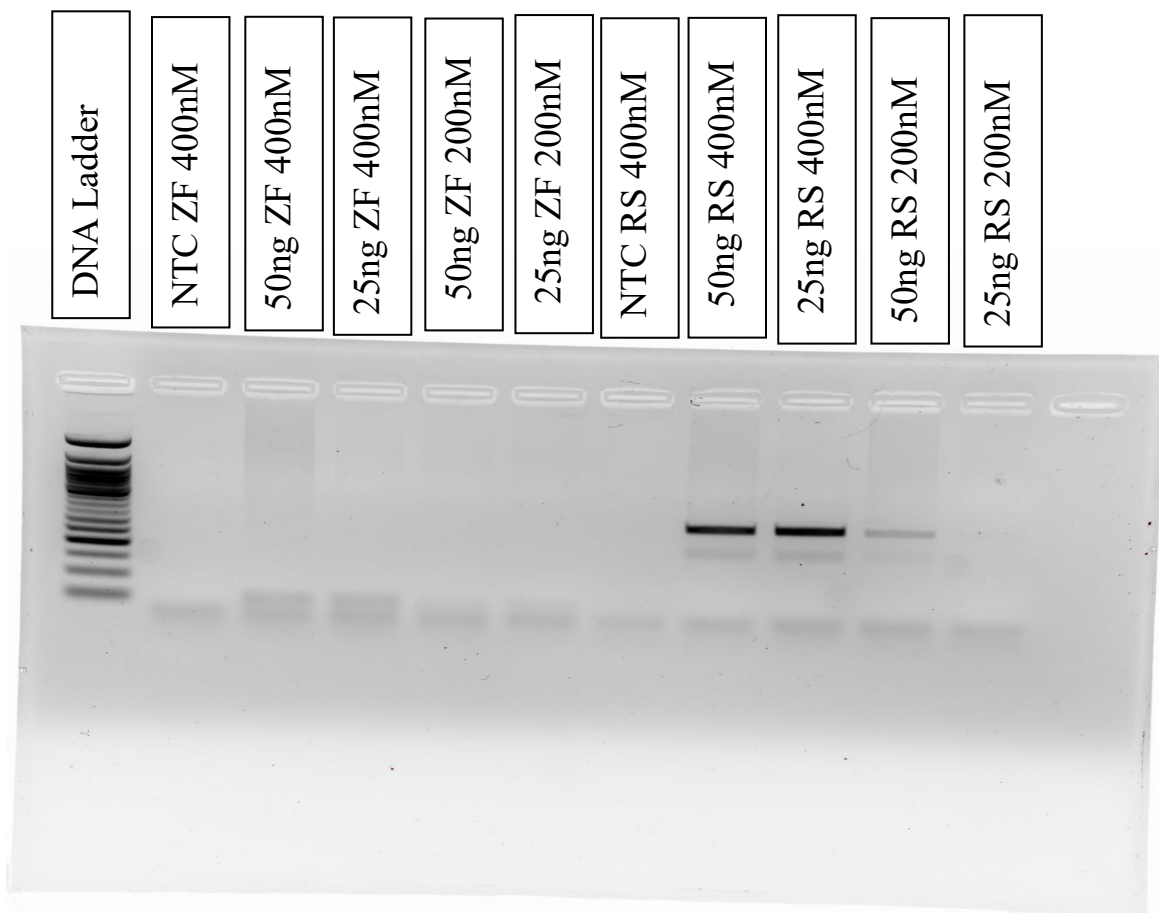


Figure 5. Gel containing products following a qPCR standard curve optimization run; 1:2 serial dilution with 4 dilution points (50ng, 25ng, 12.5ng, 6.25ng). Gel secondarily confirms RS GAPDH 400nM primers produced clearest results—clear amplicons of approximately 300bp in length.

APPENDIX B

General Stocks

Table 2.1 Telomere and GAPDH primer sequences.

Primer Name	Sequences (5'-3')	%G C
Tel1b	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	49
Tel2b	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	54
RS	TGACCACTGTCCATGCCATCAC	55
GAPDH (Forward)		
RS	TCCAGACGGCAGGTCAGGTC	65
GAPDH (Reverse)		

Table 2.2 Final 'singleplex' telomere reaction components.

Component	Stock Concentration	Final Concentration	Volume	Source of Reagent
Power SYBR®	2x	2x	12.5µl	Applied Biosystems
Green Master Mix				
Tel1b primer	100µM	900nM	1µl	Integrated DNA Technologies
Tel2b primer	100µM	900nM	1µl	Integrated DNA Technologies
Template*				
Millipore Water (MPW)**				

*Samples contained different starting concentrations and thus required different volumes to reach 10ng per reaction—Negative controls contained equivalent volumes of MPW

**Volume of reaction made up to 25µl with MPW

Table 2.3 Final ‘singleplex’ GAPDH reaction components.

Component	Stock Concentration	Final Concentration	Volume	Source of Reagent
Power SYBR®	2x	2x	12.5µl	Applied Biosystems
Green Master Mix				
RS GAPDH Forward primer	100µM	400nM	1µl	Integrated DNA Technologies
RS GAPDH Reverse primer	100µM	400nM	1µl	Integrated DNA Technologies
Template*				
Millipore Water (MPW)**				

*Samples contained different starting concentrations and thus required different volumes to reach 10ng per reaction—Negative controls contained equivalent volumes of MPW

**Volume of reaction made up to 25µl with MPW

Table 2.4 Final Telomere Cycling Profile on ABI 7500 System.

Program Number	Target (°C)	Hold	Number of Cycles
1	95°C	10:00	1
2	95°C	1:00	
	54°C	1:00	30
	60°C	1:00	

Table 2.5 Final GAPDH Cycling Profile on ABI 7500 System.

Program Number	Target (°C)	Hold	Number of Cycles
1	95°C	10:00	1
2	95°C	1:00	
	62°C	1:00	40
	60°C	1:00	

APPENDIX C

Melt/Dissociation Curve Analyses

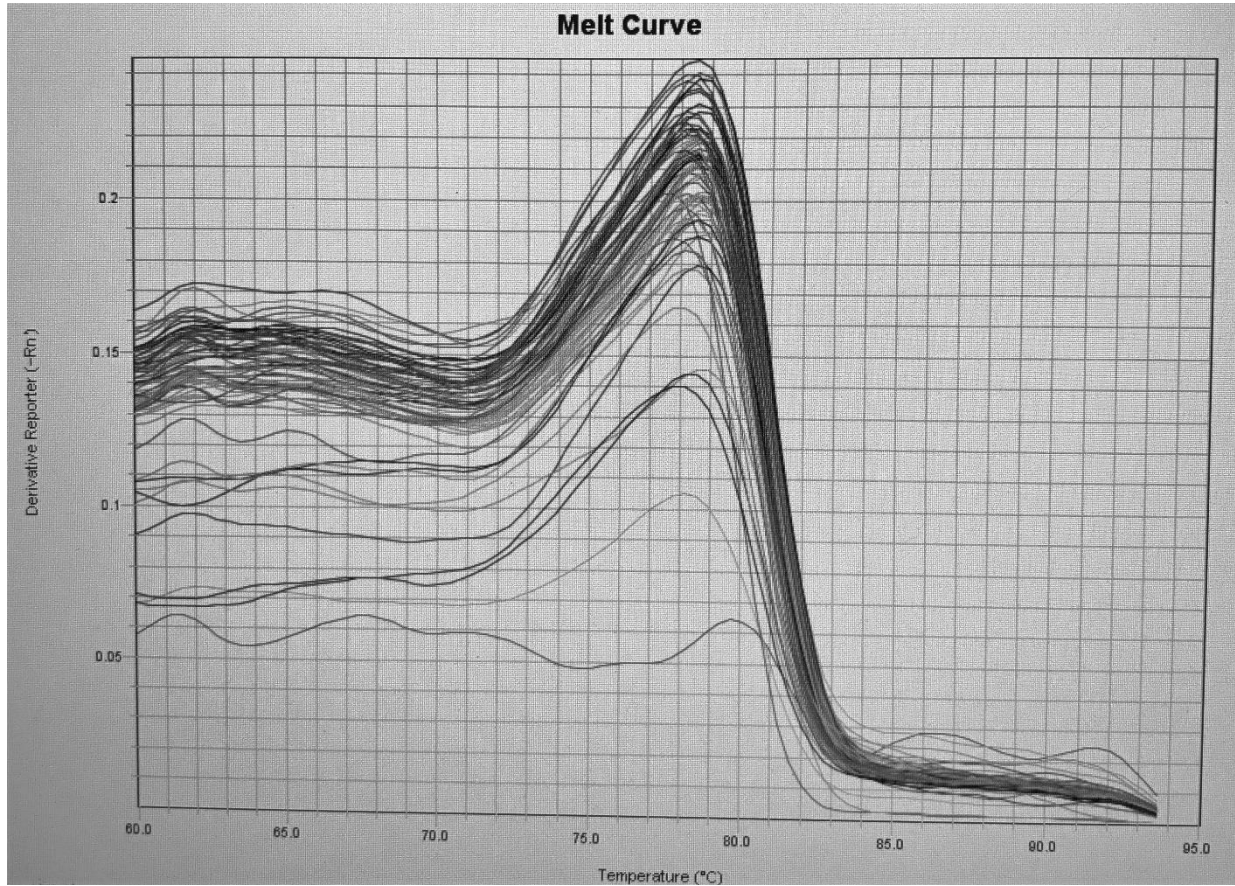


Figure 6.1. Melt curve/dissociation analysis of the first telomere (T) plate performed after amplification. Melting temperature (T_m) of amplicons confirmed at approximately 78°C; only one peak observed.

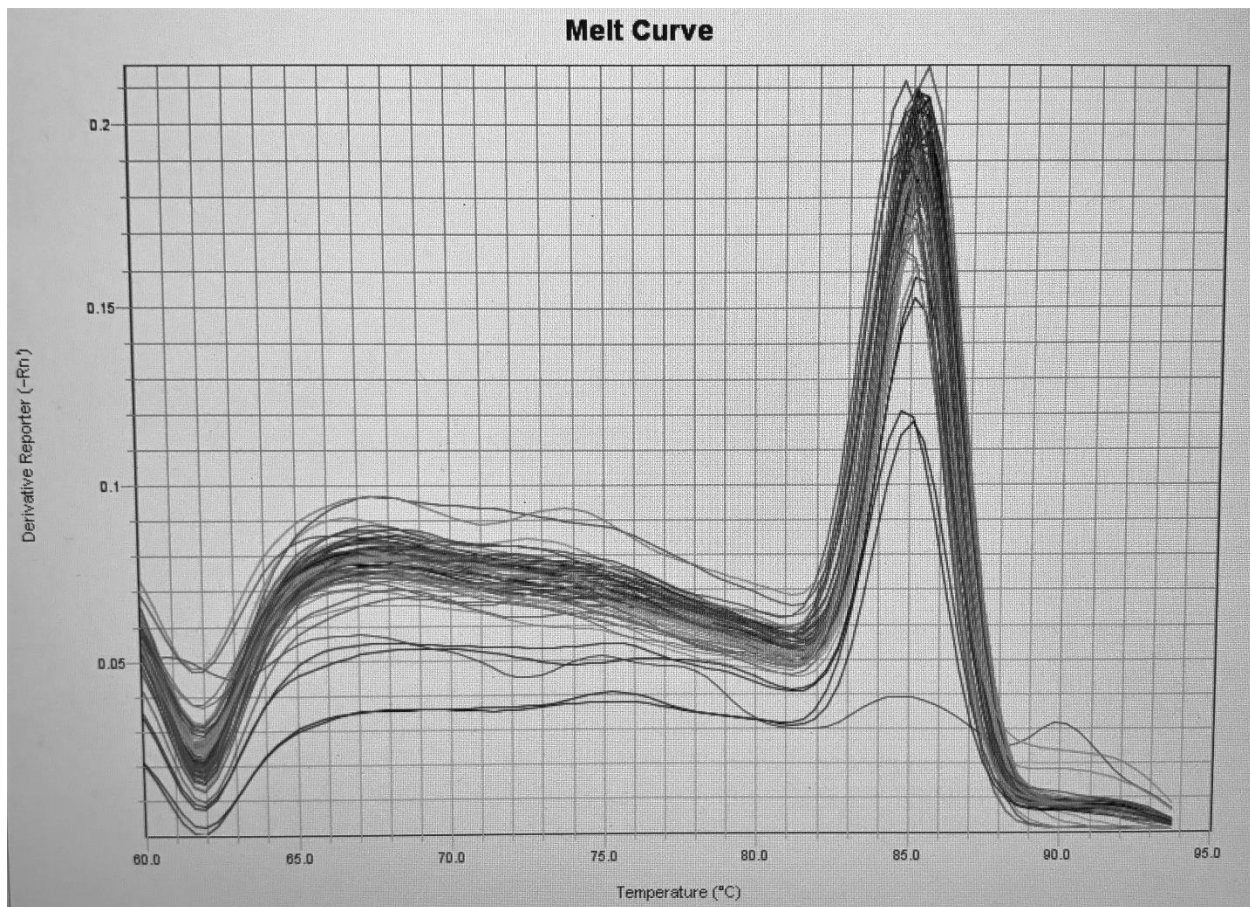


Figure 6.2. Melt curve/dissociation analysis of the first GAPDH (S) plate performed after amplification. Melting temperature (T_m) of amplicons confirmed at approximately 85°C; only one peak observed.

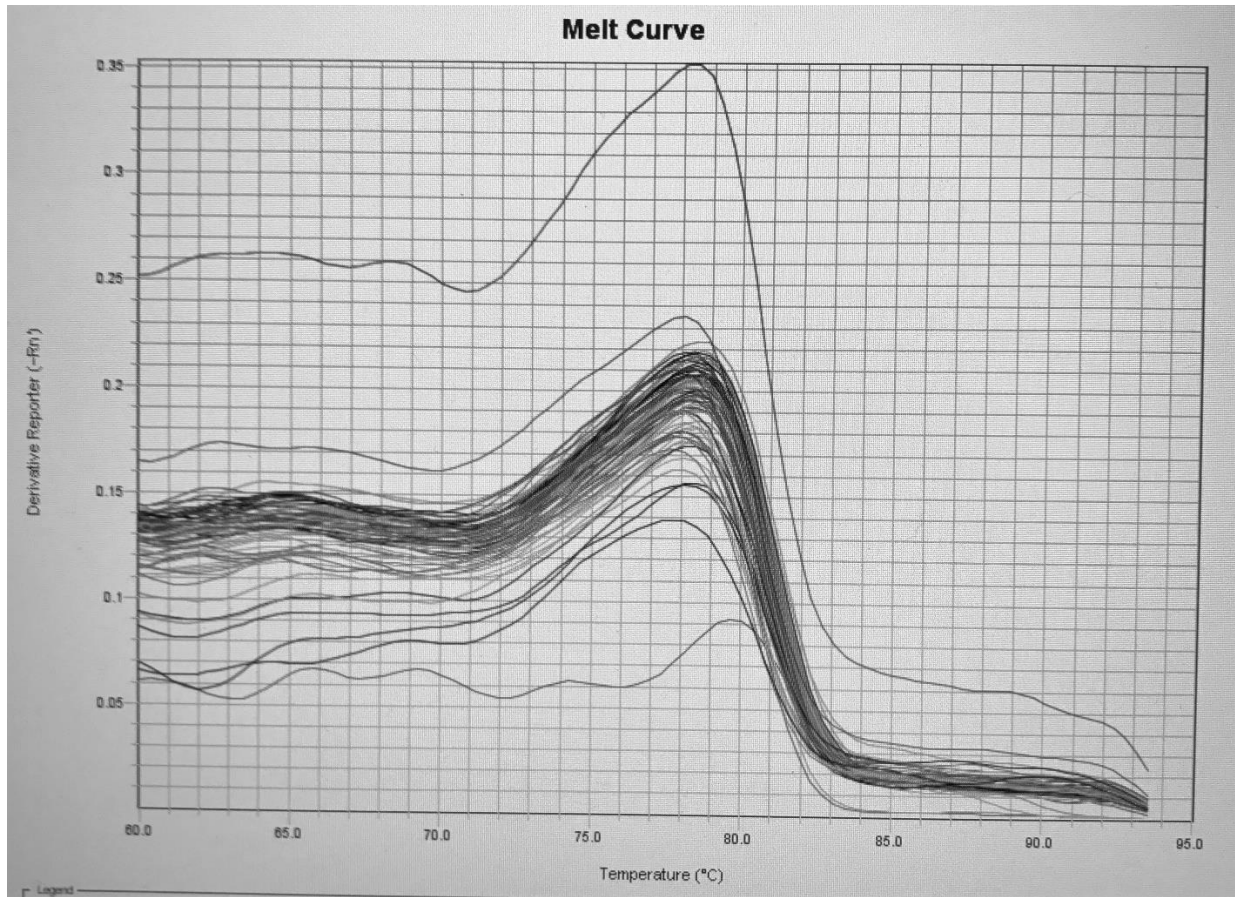


Figure 6.3. Melt curve/dissociation analysis of the second telomere (T) plate performed after amplification. Melting temperature (T_m) of amplicons confirmed at approximately 78°C; only one peak observed.

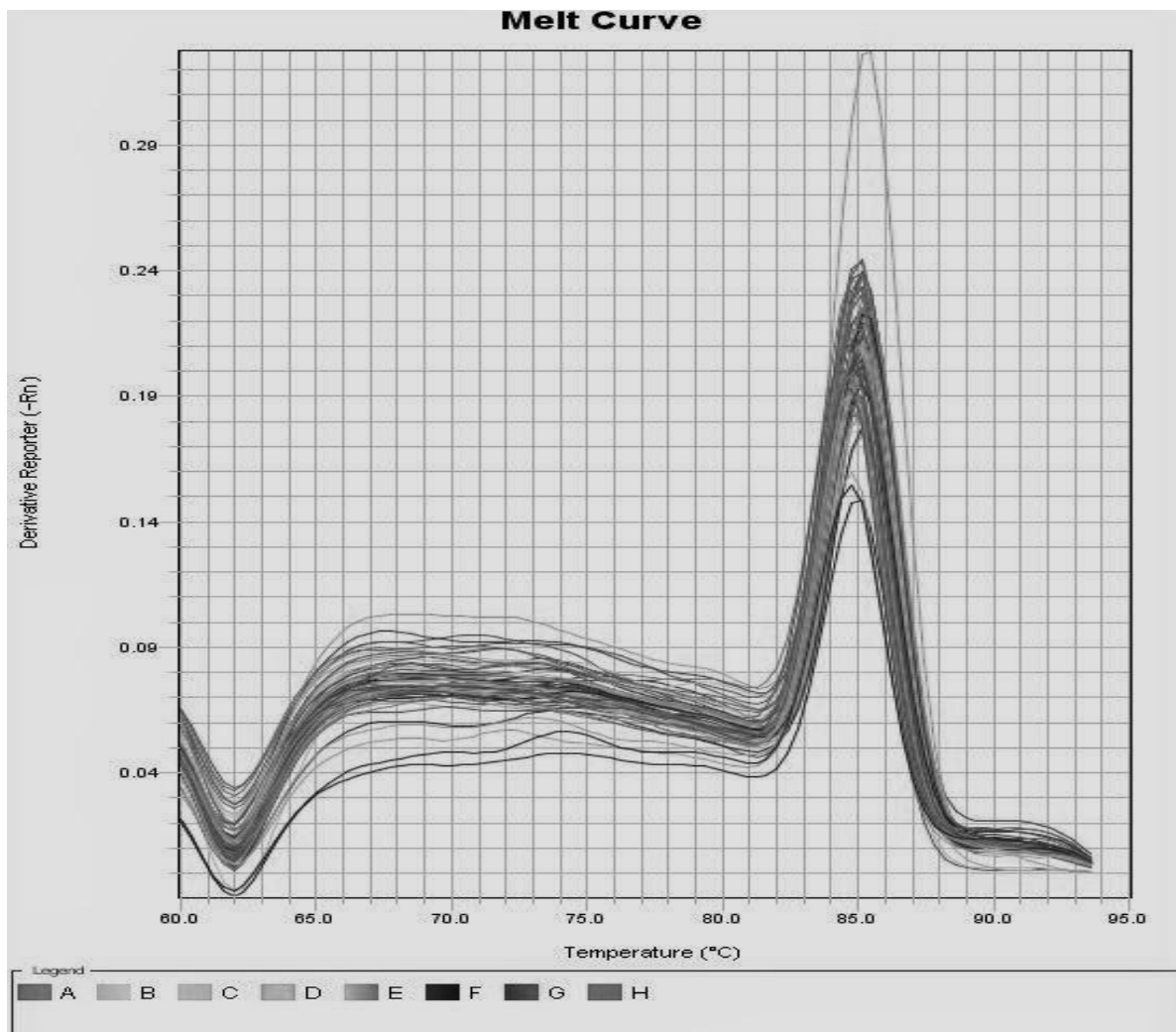


Figure 6.4. Melt curve/dissociation analysis of the second GAPDH (S) plate performed after amplification. Melting temperature (T_m) of amplicons confirmed at approximately 85°C; only one peak observed.

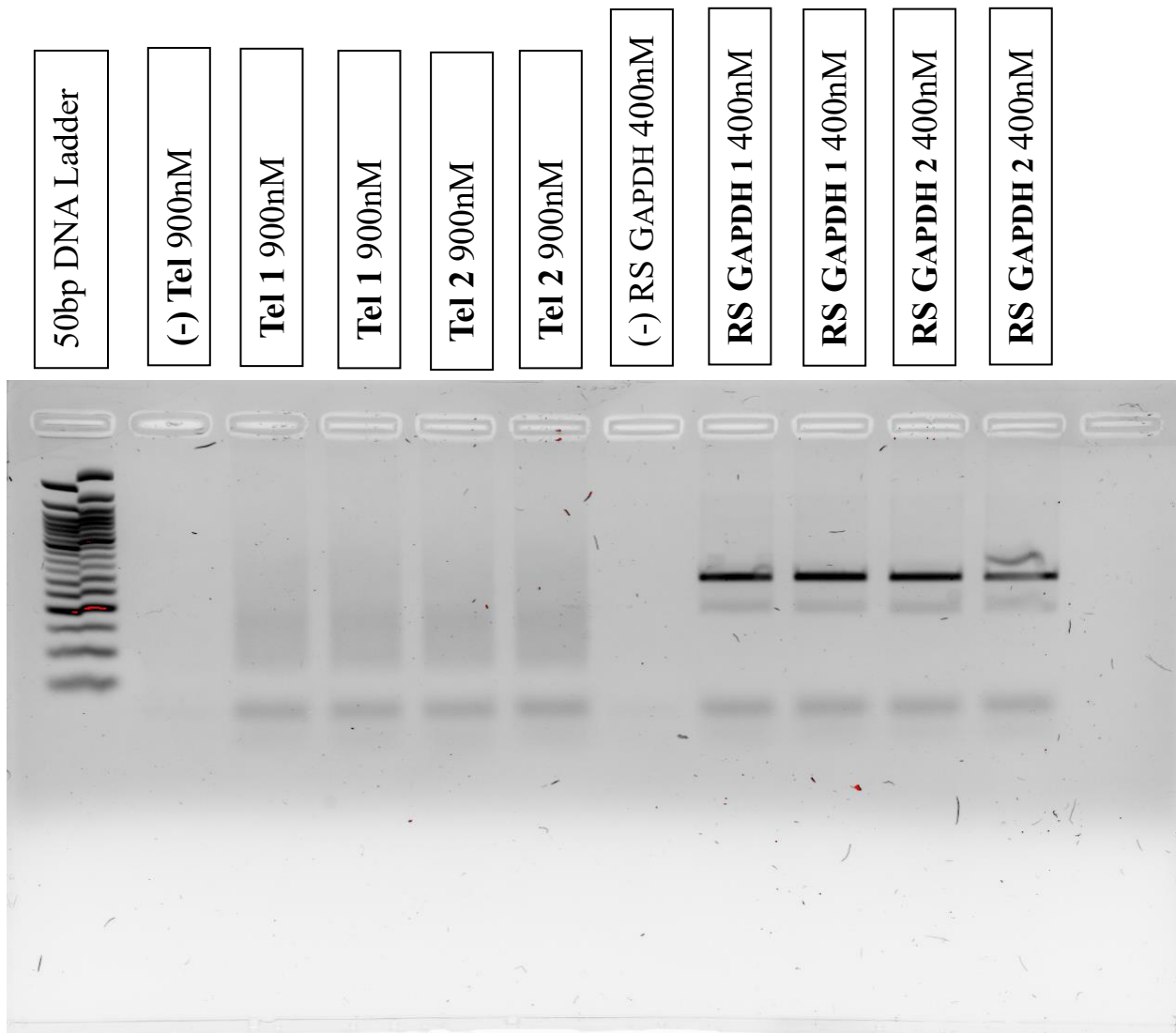


Figure 7. Gel containing two randomly chosen reaction wells from each of the duplicate telomere (T) and GAPDH (S) experimental plates after melt curve/dissociation analysis, respectively. Confirms production of expected amplicons all selected sample reactions.