Telomeres dynamics across early life of the Cuban Brown Anole (*Anolis sagrei*)

By

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A thesis submitted to the Graduate Facility of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
December 9, 2023

Keywords: brown anole, telomere, early life, insulin growth factor, sex

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Abstract

Telomere length is a key biomarker for cellular senescence and organismal survival. However, relatively little research has been conducted on the dynamics of telomeres in early life, particularly in reptiles. Through the research in this thesis, I focus on telomere dynamics in the early life stages of the brown anole lizard (*Anolis sagrei*). In my first project, I test if telomere length changes in the blood cells during the transition from embryo to 1-week post-hatching, and whether telomeres in males are females differ at this early stage. To do this I optimize and validate a molecular sexing method to identify sex of the brown anoles that yielded. I use qPCR assay to quantify telomere length in blood cells, and I tested whether telomere length changes during the transition from the embryonic stage, within 24-hour of hatching, and to one-week of age. I found that across the three time points observed that there were no significant differences in telomere length between ages. When testing for sex specific differences in telomere lengths across these time points, I also found no sex specific differences.

In the second project, I aimed to determine if the addition of insulin-like growth hormone factors (IGFs) and growth rate would impact telomere dynamics in the early life of the brown anole lizard, and if this response differed by sex. To do this, I introduce IGF-I and IGF-II through periodic injections to juvenile brown anoles and quantified their telomere length in the blood cells at 13-weeks of age. I found no significant differences across injection treatments. When I compared 13-week-olds that received IGF injection treatments to the week one, I also found no significant difference for age in telomere lengths. Lastly, I quantified growth for each individual using total snout-vent-length (SVL) change and body mass change between week 1 and week 13 to test for potential relationships between IGF treatment, growth, telomere length, and sex. I found that telomere length change in the interaction between growth and treatment, where telomere lengths decreased in both orange and yellow treatments but increase in the pink treatment. Sex was also significant in each model with males having longer telomeres relative to females.

My work described in this thesis helps to move the scientific community forward in various ways. Firstly, through this work I was able to develop a molecular sexing protocol via qPCR using primer and probe set for an autosomal gene and an X-linked gene alongside telomere analysis via qPCR. Secondly, this work provides the knowledge of telomere dynamics during the early life stages of *A. sagrei* for cross species telomere analysis. I show that telomeres lengths do not vary in male or female *A. sagrei* in embryonic stage and at one week of life, but males did have longer telomeres at 13 weeks of life. Further research is needed to understand how telomeres change as the animals continue into adulthood. These findings would provide foundational work to expand upon to better understand telomere dynamics in a model reptile species in future studies.

During my time at Auburn University, I have had many more contributions to the scientific field outside of my thesis experimental work. A large portion of my efforts have gone toward to collection, processing of 1,600 *A. sagrei* eggs, and subsequent care of the animals for a NIH-funded project to develop a new reptile model for sex-specific aging. Periodic collection of biometrics for the 1,200 individuals such as SVL, body mass, back pattern photos and bite force. Collection and preservation of fine tissues including liver, heart, brain, skeletal muscle, and blood tissue for several hundred *A. sagrei*. Various primer and probe set designs and optimization for molecular sexing to be performed on other species such as brown anole lizards, zebra finches and alligator lizards. Additionally, I helped train many undergraduate researchers in both animal care and data collection procedures and molecular laboratory techniques.

Acknowledgements

I would like to start by noting my appreciation for Dr Tonia Schwartz for giving me the opportunity to pursue this master's degree under her guidance and mentorship. I am grateful for the numerous scientific skills that I have gained that better prepare me to reach my goals in a professional career as well as the opportunity to mentor other young scientists. I want to thank my committee members Dr. Tonia Schwartz, Dr Haruka Wada, and Dr, Daniel Warner for all their time and guidance throughout this lengthy process. To my lab mates past and present, I could not have completed this body of work without the support that I received from the Schwartz Lab. From the early morning discussions of how to better optimize protocols to the late-night coding sessions, I will always be appreciative of that time given. I can say that my time working in this lab and the friendships developed within the lab have made me a better professional and individual overall.

To my father, I cannot express enough the appreciation I have for the values and ethics that you instilled in me from an early age. Your constant support of me bettering myself throughout the years cannot be repaid. To my stepmother, I am forever grateful for you filling the role of my mother for so many years. I will always appreciate your kindness and selflessness. I want to thank all of my friends for the years that you have all been a part of the support system that has helped me continue toward the things I want in life.

I want to thank NIH-R15 grant "A new vertebrate model to study the role of growth factors IGF1 and IGF2 in sex dimorphism of longevity and aging" and Auburn University for funding my work.

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Chapter One: Telomere length dynamics in the transition from embryo to early life in a model reptile

This chapter will be submitted for publication, after the integration of an undergraduate student's research project that is in the final stages.

Hardin, Payne, and Schwartz. Telomere length and dynamics in the transition from embryo to early life in a model reptile.

Introduction

Telomeres are the dynamic, short tandem repeats comprised of highly conserved DNA sequences that are located at the ends of chromosomes (Blackburn 1991, Rhodes and Giraldo 1995). They serve as protective caps at the ends of the chromosomes and function to assist in chromosome stability to prevent genetic fragility and cancer development (Hermann et al 2001). Telomeres have become widely known as key biomarkers for cellular maintenance and aging (Sanders and Newman 2013). Telomeres have been studied extensively in the context of aging with a general trend observed across species where telomere length will steadily decline as species age (Remot et al. 2022). While this trend is widely observed across species, telomeres are relatively understudied when looking at telomere dynamics in early life.

Various studies have been conducted across species that show telomere attrition as individuals age. This trend has been observed in mammalian studies in human fibroblast cells that showed telomere length attrition due to aging and cell division (Harley et. al, 1990). These findings are supported by additional studies using mice comparing young female oocytes to reproductively aged female oocytes that found the reproductively aged female oocytes had shorter telomere lengths (Yamada-Fukanage et al. 2013). In Australian sea lions a decline in telomere length was also observed but only after the juvenile stage of develpment(Izzo et al. 2011). In avian species, a study in birds showed that both long lived species and short-lived species experience consistent telomere length attrition, however long-lived species experience less telomere length attrition by comparison (Tricola et al. 2018). One study in the long-lived species loggerhead sea turtle showed tissue specific telomere length attrition in the epidermis as individuals age (Hatase et al.

2008). A study in the American alligator also showed that telomere length was negatively correlated with body length alluding to the oldest individuals also having the shortest telomere lengths within the population (Scott et al. 2006). However, a study in frilled neck lizards found that telomere lengths would be longest on average during the midlife stage of the lizard (Ujvari et al. 2017). Lastly Ujvari and Madsen observed a different trend was in pythons showing that there is no significant decrease in telomere length from 1 year of age to 9 years of age. This was observed between male and female pythons, with sex specific differences being present showing females having longer telomere lengths (Ujvari and Madsen 2009). With the previously mentioned studies showing large variations in telomere dynamics across on organism's life span, it can be seen that telomere dynamics are highly variable. These differences in telomere dynamics across species could be attributed to variation in telomerase activity across species. Telomerase is the enzyme that repairs or elongates telomere lengths by adding nucleotides to the telomere sequence. Telomerase activity can help prevent the overall telomere lengths from senescence and ultimately reaching a critical length (Preston 1997).

When looking at the python study by Ujvari and Madsen, although it is not a longitudinal study, an increase in telomere length was observed between year zero and year one and was found to reach the peak telomere length by year one. The increase in telomere length found between early life points would suggest a large telomere length spike within that time period. With no data for telomere lengths within this first year of development, it is unknown when this telomere length increase takes place. Additionally, if sex specific differences are present in the species of study, at what point would sex specific differences be evident. This presents a gap in knowledge regarding early life telomere dynamics in pythons or more broadly, reptiles.

To address this gap in knowledge, I propose using *A. sagrei* as a model organism to quantify telomere length at three times points from the embryonic stage to one week of age. The embryonic stage telomere analysis would illuminate telomere dynamics at the earliest time point for *A. sagrei* and allow for comparisons across the other time points to determine if changes in telomere length occur within the first week of life.

To test for sex specific differences between female and male samples and across treatments, a molecular sexing protocol was be used to validate the sexes of each individual. This method was validated using two separate groups of known female and male *A. sagrei*.

The goal of this study is to understand the telomere dynamics associated with specific time points during early life of brown anole lizards (*Anolis sagrei*) by addressing the following objectives:

1) Develop molecular sexing protocol for the brown anole and test for sex specific differences in telomere length in early life.
2) Determine if telomere length changes in the transition from embryo to one week of life in the brown anole.

The study will help to fill a gap in knowledge by illuminating early life telomere dynamics for a model reptile. Moreover, the importance of understanding telomere dynamics in reptiles is to develop a comparative model to understand what differences occur across reptiles, mammals, and birds in regulation of telomeres.

Materials and Methods

Experimental design: In the summer of 2020, eggs were collected from the Schwartz Lab breeding colony at Auburn University (IACUC PRN: 2019-3521). The eggs were incubated in petri dishes (VWR International, 60x15mm, Cat # 25384-090) that contained 1 tbsp of 50:50 mass ratio water/vermiculite. Each egg was assigned an "AA" number that was recorded for identification throughout experimental processes. The petri dish was labeled with the "AA" number printed on a sticker (Avery Multiuse Removable Round Labels, Product #6450) along with the egg collection date to predict the hatch dates of individuals. The Petri dishes were wrapped in 2" strips of parafilm (Bemis Parafilm "M" PM-999/ 4inx250ft roll) to prevent desiccation during incubation, and later wrapped twice with 2" strips of parafilm due to moisture loss from cracks developing in the single layer of parafilm. Eggs were assigned to three age

groups that determined when the samples would be collected: (1) Embryo, embryos at day 27 of incubation that are 1-2 days from expected hatch date at stage 18 (Sanger et al. 2008); (2) Day One, hatchlings within 24 hours after hatching; and (3) Day Seven, hatchlings at 7 days of age.

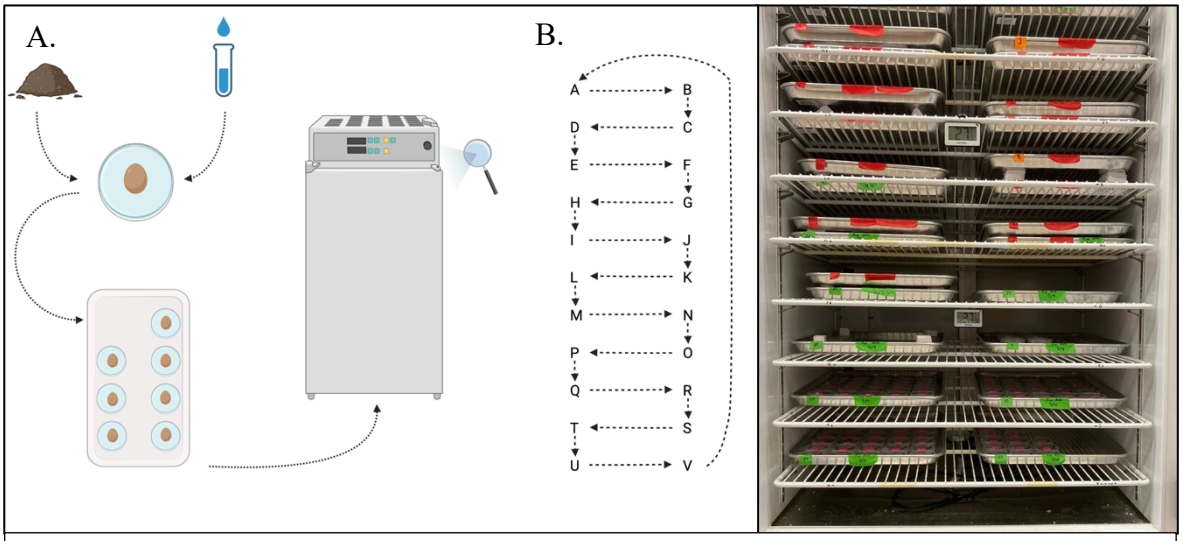

Figure 1.1. Egg processing and incubation rotation system.
A. Diagram showing how each egg was processed and placed on a baking tray before being placed into the incubator.
B. Representation of the rotation schedule in order for each egg to experience the same temperatures throughout the incubation process up to hatching, unless designated to the "embryo" treatment.

Once egg processing was complete, the eggs were placed in an incubator (Percival, I36NL) at 27°C. The incubator had 11 shelves total with room for two metal trays side by side (Baker's Mark, half size perforated pan, measurement "0.054 M3") making the total number of tray positions 22. When all 22 positions were occupied by trays of eggs, trays would be stacked two high with small squares of 2" x 2" foam placed in each corner between the petri dish and the tray above them to prevent petri dishes from sticking to the tray placed on top. The eggs from the most recent collections would be stored underneath previously collected eggs to prevent missing a hatchling during the routine checks for new hatchlings. Each rack had two positions indicated by letters. This was to ensure that each egg would receive the same temperature exposure by rotating the trays through the incubator, changing one position every Tuesday, Thursday, and Saturday.

Eggs were checked daily for hatchlings. If an individual designated to the embryo treatment hatched before its dissection date, it would then be reassigned to either the Day One or Week One Groups. To replenish the power for the embryo treatment, the reassigned individual would be replaced with another that has yet to hatch from a treatment the new hatchling was reassigned.

I collected various tissues (for other projects) and blood samples, via dissection, from 30 individuals from each of three age groups. In preparation for dissections, 1.5ml screw cap tubes (Thermo Scientific Screw Cap Micro Tubes, 1.5ml, Ref# 3474) were labeled with the "AA" ID as well as an abbreviation of "BL" that corresponds to the blood tube containing 15ul of DNA/RNA shield (Zymo Research) to allowing for 2 years of DNA preservation at 4°C. Equipment used in dissections included dissection boards (Simport, M630-1 DispoCut Dissecting Board, 152 x 203mm), dissecting pins (Dritz Home 214500 Bulk Package of Nickel-Plated Steel T-Pins, 350-Pack, Silver), scalpel, fine dissection scissors, forceps, and butcher scissors for euthanization. Before dissections began and in between each individual dissection, all tools were disinfected by rinsing them in DI water, followed by 10% bleach, rinsing in DI water, RNAse away, and a final rinse in DI water. The blood was collected from the decapitation site with a heparinized capillary tube (KimbleChase, cat. no. 2501) and ~5 µl was mixed with the 15 µl DNA/RNA shield and then stored at 4°C. Sample sizes for each treatment varied slightly with Embryo n=18, Day On=20, and Day Seven n=23 (Table 1.1).

Figure 1.2 Figures of experimental time points. From left to right: Embryo, Day One, and Day Seven.

DNA Isolations

In preparation for DNA isolations, all samples were randomized in order of isolation to prevent biases across day of DNA isolation affecting experiment groups. I conducted DNA isolations from 5ul of the 5ul/15ul blood to DNA/RNA shield mixture and using a Gentra Puregene DNA Isolation Kit A (Qiagen) "Cultured Cells" protocol provided by the manufacture. Gel electrophoresis was conducted to assess DNA quality by loading 5ul of DNA on a 1% agarose gel (0.5gm agarose in 50 ml TAE) running at 100V for 1 hour. DNA was quantified using Accuclear Ultra High Sensitivity dsDNA Quantification Kit (Biotium) running triplicates on a 96 well plate on the Cytation 3 imaging reader (BioTek) with the protocol calling for 3ul of standard and 2ul of DNA with DPEC water to equal out to 10ul per well. A dilution of 5 ng/µl was made for every sample to standardize the concentration of DNA for the telomere analysis and sex validation.

QPCR Analysis of Molecular Sexing and Quantifying Telomeres.

The process of performing qPCR analysis for this experiment was split into two separate qPCR types of reactions per individual. The first qPCR reaction was a probe-based multiplex for the sexing validation protocol that included a single copy nuclear autosomal gene that was for sexing and for normalizing the telomere reaction. The second qPCR reaction was dye-based telomere qPCR reaction being ran on the same day. Each of these reactions per individual were run in triplicate on the same 96-well plate. A total of 24 individuals and a set of Standards and Controls were run in triplicate on a plate, and total of sets of plates were run, for a total of 96-well qPCR plates within three days to gather all the data needed for analysis. During the second qPCR plate reaction there was a user error in loading the standards where the first standard did not get into the reaction well causing the expected dilution series of the standard to not be present. This was corrected by manually adjusting the expected dilution series for that one plate, using all plate standard data for experimental analysis, and using a Gblock for the standard curve (described below).

Preparing the qPCR Absolute Standard Curve.

Two DNA standards were developed for the qPCR analyses. First, a was designed to hold target X chromosome and single copy autosomal genes (Figure 1.3). Second a 72bp oligo that was designed to contain 13 telomere repeats of "TTAGGG" (Table 1.1). The sequences in fasta format are included as an appendix. These were pooled to create a single standard, that was used to make a 6-point,10-fold dilution series to create an absolute standard curve as described in detail below.

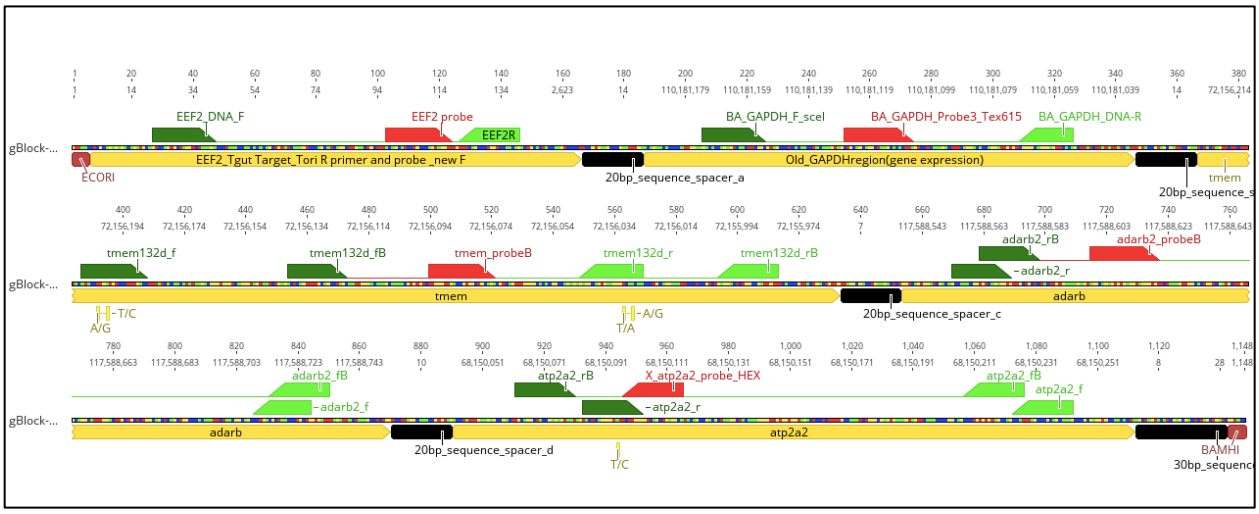

Figure 1.3 Gblock design

The Gblock was designed to be used by various projects and included PCR target regions from eef2 from zebra finch, and the following from brown anole reference genome (Geneva et al 2022): autosomal single-copy genes gapdh and adarb2, and X-linked genes tmem132dand atp2a2. The target genes used for molecular sexing of the brown anoles in this study were the autosomal genes adarb2 and X-linked gene atp2a2. The selection of these genes for molecular sexing was taken from the methods of Rovatsos and Kratochvil (2016). The autosomal gene adarb2 also serves as our single-copy gene for the telomere analyses.

Both standards were ordered to be manufactured by Integrated DNA Technologies, and they were shipped lyophilized.

The Gblock and the Telomere Oligo were resuspended using 1X TE (Tris, EDTA) (IDT Cat #) to 7.948x10^11 copy numbers for the Gblock (resuspended with 1,000ul TE), and 6.561x10^15 copy number for the Telomere Oligo (resuspended with 2,000ul TE). Low bind 1.5 ml tubes (VWR cat. no. 76332-068) were used to hold the Stock standards. For Standard 1, 12.58 ul of Gblock stock, 3.05 ul Telomere Oligo stock, and 84.37 of Vector-spiked water. This would provide a Standard 1 with a concentration of 100,000,000 copies/ul that would then be diluted ten-fold in the Vector-spiked water to make a standard series consisting of seven concentrations. Standards were aliquoted (10 µl each) into PCR strip tubes (cat. no. 93001-118) and stored at -20C for single use to avoid freeze-thaw.

Table 1.1 Details for the qPCR primers and probes. The telomere primers were originally published in Cawthon (2002). The sexing primers were redesigned from of Rovatsos and Kratochvil (2016) to target the same gene regions in brown anoles.

Primer/Probe	Sequence	Tm	PCR Product Size (bp)
Telg_Sethi2021_F	**5' ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T 3'**	**66.7C**	**40bp**
Telg_Sethi2021_R	**5' TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA 3'**	**64.4C**	**42bp**
Adarb2_fB	**5' TTA CTC CTG CTG GGA ATG CG 3'**	**57.4C**	**20bp**
Adarb2_rB	**5' AGA CTG TGG AGA AGC AGC AC 3'**	**57.2C**	**20bp**
Adarb2_probeB_Cy5	**5' /5Cy5/ TGG CTG TGA /TAO/ GAG TGG ATG GGA GA/3IAbRQSp/ 3'**	**61.8C**	**23bp**
Atp2a2_fB	**5' TAG GCA GGC ATC TCT CTG GG 3'**	**58.6C**	**20bp**

Atp2a2_rB	**5' AGC TGG CAT CCG TGT GAT TA 3'**	**56.6C**	**20bp**
X-Atp2a2_probeB_HEX	**5' /5HEX/ AGC CAC TGC /ZEN/ AGT CCC CTT GT /3IABkFQ/ 3'**	**61.7C**	**20bp**
Telomere13_RE_STD	**5' GGA TCC TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG AAT TC 3'**	**70.8C**	**89bp**

Validation of Molecular Sexing Method in Brown Anoles

The development of the molecular sexing protocol for brown anoles was necessary to validate the sex of the embryos and hatchlings used in this study. In the Florida population that is the source of the study animals, male *A. sagrei* can only have a chevron back pattern, the female counterpart can have multiple morphs of the back pattern including the chevron pattern indicative of a male (Feiner et. al. 2022). While visual methods such as post anal scale identification can be effective in sex validation in older juveniles and adults, it can be inconsistent based on developmental stage of observation and ability to visually discern post anal scales. With these barriers, sex cannot be identified with 100% certainty by visual inspection at these early stages. With *A. sagrei* having XX/XY sex determination, a multiplex qPCR protocol was developed to quantify copy number for an X-linked gene (atp2a2) relative to an autosomal gene (adarb2) based on the methods from Ravotsos and Kratochvil (2016). Primer sets to amplify the genes atp2a2, adarb2, tmem132d, and gapdh, along with a probe were redesigned to complement the *A. sagrei* genome (Geneva et al. 2022) and validated via gel electrophoresis after PCR to ensure each set of primers produced the expected PCR product size (Table 1.2). After validating the primer sets amplified the correct size target, complementary qPCR hydrolysis probes (Table 1.1) were ordered (Integrated DNA Technologies) for each primer set and tested via qPCR and high PCR efficiency was confirmed based on the Absolute Standard Curve.

The multiplex qPCR was run in triplicate in 96-well white-walled plates (BioRad cat. no. HSP9655), with BioRad Microseal 'B' seals (cat. no. MSB1001). The 20ul volume reaction consisting of 10ul (1X) of IDT PrimeTime Gene Expression 2x Master Mix, 0.4ul of each Adarb2 and Atp2a2 primers and probe (all at a final working concentration of 10 μM), 5.6ul of molecular grade water, and 2ul of 5ng/ul of DNA template per reaction. The following thermal cycle conditions were used on a BioRad CFX96 Optics Module qPCR machine using the Cy5 channel to detect the fluorescence of Adarb2, and the Hex channel to detect the fluorescence of Atp2a2. Thermal Cycle was as follows. Stage 1: a single cycle of 95°C for 3:00 min; Stage 2: two cycles of 95°C for 0.15s, 49°C for 0.30s, 55°C for 0.30s; Stage 3: thirty five cycles of 95°C for 0.15s, 56°C for 0.30s, 60°C for 0.30s, plate read; Stage 4: melt curve 60°C to 95°C increment 0.5°C 0.05s plate read. Note: Stage 2 and Stage 4 are necessary for the Telomere amplification, but not for this molecular sexing. This protocol works equally well for molecular sexing if you delete these stages. The absolute standard curve was also run in triplicate on every plate and was used to calculate the starting quantity of target genes, which represents the number of those chromosomes in the reactions.

The primary objective of this protocol was to enabled testing for the X-linked gene copy number in relation to the autosomal gene copy number. QPCR was conducted on a training set of known males (N=12) and known females (N=12) and the starting quantity of the X-linked gene was divided by the starting quantity of the autosomal gene. These X-linked gene/autosomal gene ratios from the training set produced distinct ranges for each sex resulting in a male X/A range of 0.45-0.65 and a female X/A range of 0.85-1.05. We validated these ranges for defining sex using a test set of a different known males (N=12) and known females (N=12). This protocol was used to validate the sex of all individuals to test for sex specific differences in telomere length across experimental age groups.

qPCR Sex Identification and Telomere Length Quantification.

The 61 individual samples were randomized to plates to be run in triplicate, with standard curve, known male and female controls, and a No Template PCR control on every plate. Every set of 24 samples had a "Sexing Plate" to collect the data for the X-linked and Single-copy Autosomal

Gene as described above, and a "Telomere Plate" for collection of the telomere data as described below. These two plates were prepared and run sequentially on the same day using the same aliquot strip-tube of Absolute Standards and IDT master mix, and same dilutions of samples.

Using methods from Sethi (2020) and Cawthon (2009), we were able to determine telomere lengths using dye-based qPCR to measure the telomere quantities of our experimental samples. The telomere qPCR was run in triplicate on 96-well white-walled plates (BioRad cat. no. HSP9655), with BioRad Microseal 'B' seals (cat. no. MSB1001). The 20ul volume reaction consisting of 10ul (1X) of IDT PrimeTime Gene Expression 2x Master Mix, 0.6ul (final concentration 10um) of telomere primers from Cawthon 2009 5.8ul of molecular grade water, 1ul of 20X (final concentration 1x) Biotium Evagreen+ (cat. no. 89138-996), that binds the double-stranded PCR products to fluoresce), and 2ul of 5ng/ul of DNA template per reaction. The following thermal cycle conditions were used on a BioRad CFX96 Optics Module qPCR machine using the SYBR channel to detect the fluorescence of the Evagreen representing the amount of PCR product of the telomere repeats. Thermal cycle was as follows. Stage 1: a single cycle of 95°C for 3:00min; Stage 2: two cycles of 95°C for 0.15s, 49°C for 0.30s, 55°C for 0.30s; Stage 3: thirty five cycles of 95°C for 0.15s, 56°C for 0.30s, 60°C for 0.30s, plate read; Stage 4: melt curve 60°C to 95°C increment 0.5°C 0.05s plate read. The absolute standard curve was also run in triplicate on every plate and was used to calculate the starting quantity of telomere repeats.

Once all sexing qPCR data had been collected the thresholds for both Cy5 and HEX fluorescent channels was adjusted to the same position on the Y-axis across all sexing plates in order to have consistent quantification cycles or Cq values across samples and reduce potential variation from plate to plate. All individuals were then checked for outliers within the three replicates of each fluorescent channels by taking the average Cq value of an individual's replicates and subtracting the Cq value of each replicate from that average. If the difference between a replicate and the average was greater than 0.3, that replicate would then be removed from the analysis for the molecular sexing data, if an individual had more than one of the triplicates removed from either gene the whole individual was removed from the molecular sexing dataset. This procedure resulted in one individual being removed from the experiment completely and three more

individuals losing one of three replicates. Once the outliers were removed from the analysis the molecular sexing ratio was determined for each individual by taking the ratio of the average starting quantity of the X-linked gene divided by the average starting quantity of the autosomal gene. A range for males (0.41-0.66) and females (0.82-1.02) was then determined using the ratios calculated from the known males and females on the plates. Once the ranges were determined they were then applied to all unknown samples resulting in sex assigned for all but five individuals with molecular sexing ratios falling between 0.66-0.8. Sex for these individuals were left as missing data.

The telomere data processing followed the same method used for the molecular sexing qPCR data, the thresholds for the SYBR fluorescent channel were adjusted to the same position on the Y-axis across all telomere plates in order to have consistent quantification cycles or Cq values across samples and reduce potential variation from plate to plate. All individuals were then checked for outliers within the replicates of molecular sexing qPCR plates by taking the average Cq value of an individual's replicates and subtracting each replicate from the average. If the difference between a replicate and the average was greater than 0.4 cycles (Cq), that replicate would then be removed from the telomere analysis. The rationale behind increasing the room for error between replicates in the telomere protocol vs the molecular sexing protocol is that probe-based qPCR reactions are often much more consistent in comparison to that of SYBR (or EvaGreen) based qPCR. Removing all replicate outliers resulted in one individual being dropped from the experiment (same individual noted above in sexing validation) and thirteen individuals having one of the triplicates removed. After all outliers are removed, for each individual telomere quantities were normalized by the single-copy gene by taking the average Cq of telomeres dividing them by the Cq of the single-copy autosomal gene. This would tell us the approximate number of telomere repeats per genome copy.

Statistical Analysis

All statistical analysis were conducted in R using packages tidyverse, nlme, lme4, lmtest, bestNormalize, MuMin, emmeans, ggplot2, ggpubr, and devtools. The code is attached as an

appendix. To test if the qPCR the reference gene was normally distributed and to log transformation to make it normal, we used the bestNormalize package (Peterson 2020). After normalization through ordered quantile normalizing (orq) transformation, the reference gene was then tested to the copy number of the reference gene differed between these experimental variables: experimental group, sex, and age using linear models in R with the lme function in the lme4 R package. We found no statistical effect of these explanatory variables, so we proceeded to use the reference gene to normalize the telomere copy number. We then tested for normal distribution of the experimental value of telomere repeats per reference gene copy and normalized those values via orq transformation.

I used these data to address if there are sex specific differences in telomere lengths in the transition from embryo to one week of life in the brown anole. To do this, I conducted a linear model with the lme function in the lme4 R package using the explanatory variables: sex and experimental group and their interaction.

Results

Molecular sex was successfully validated by developing a ratio of the gene expression from Atp2a2 (X-linked) and Adarb2 (Autosomal) genes from known male and female *A. sagre*i DNA samples (Figure 1.3). The molecular sex for 59 of the 60 embryo and hatchling samples was able to be determined (Table 1.2).

Table 1.2 Samples separated by sex

Treatment	Female_Total	Male_Total	Sample_Total
Embryo	9	9	18
Day_1	13	11	24
Day_7	14	4	18

When conducting the molecular sexing protocol, we were successfully able to develop two distinct groups of X-linked gene copy to autosomal gene copy ratios (samples in triplicate) (Figure 1.4).

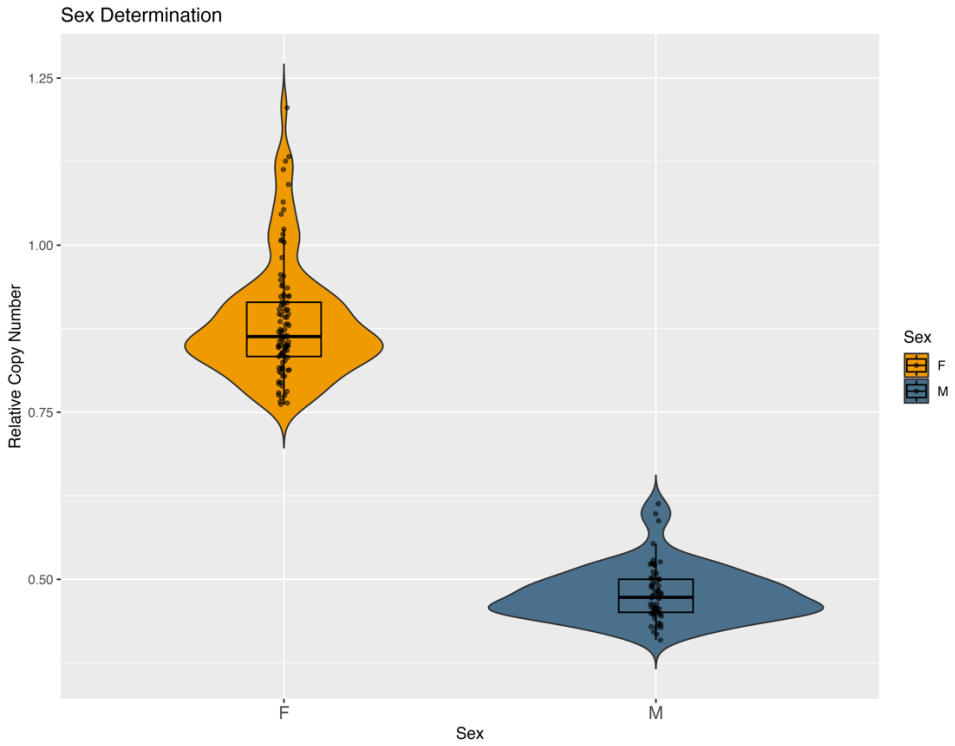

Figure 1.4 Molecular Sexing Results.

The Y-axis is the ratio of the starting copy number of the x-linked gene (Atp2a2) relative to the starting copy number of the autosomal gene (Adarb2). The ratio determined by dividing the sq of the x-linked gene by the autosomal gene resulted in two distinct groups for sex. The male brown anoles (M) will have one-half as many x-linked gene copies due to being XY and females (F) being XX. Using these two distinct groups, sex was able to be validated to test for sex specific differences throughout the experiment.

When testing for individual effects of experimental group and sex on telomere lengths, data analysis showed there was no statistical significance in telomere length when comparing telomere across the Embryonic, Day One, and Day Seven treatment groups (p-value = 0.702) (Figure 1.4, Table 1.3). When comparing telomere lengths across male and female *A. sagrei* it

was also found that telomere lengths had no significant difference (p-value = 0.707) (Figure 1.4, Table 1.3).

Table 1.3 Individual effects of Experimental Group and sex on relative telomere lengths

Column1	Column2	Column3	Column4
Statistical Test Group	Comparison	Std. Error	P-value
Treatment			0.702
Sex			0.707
Treatment x Sex			0.673
Day_7	Day_1	0.318	0.805
Embryo	Day_1	0.309	0.506
Sex_Sq_Male	Sex_Sq_Female	0.268	0.707

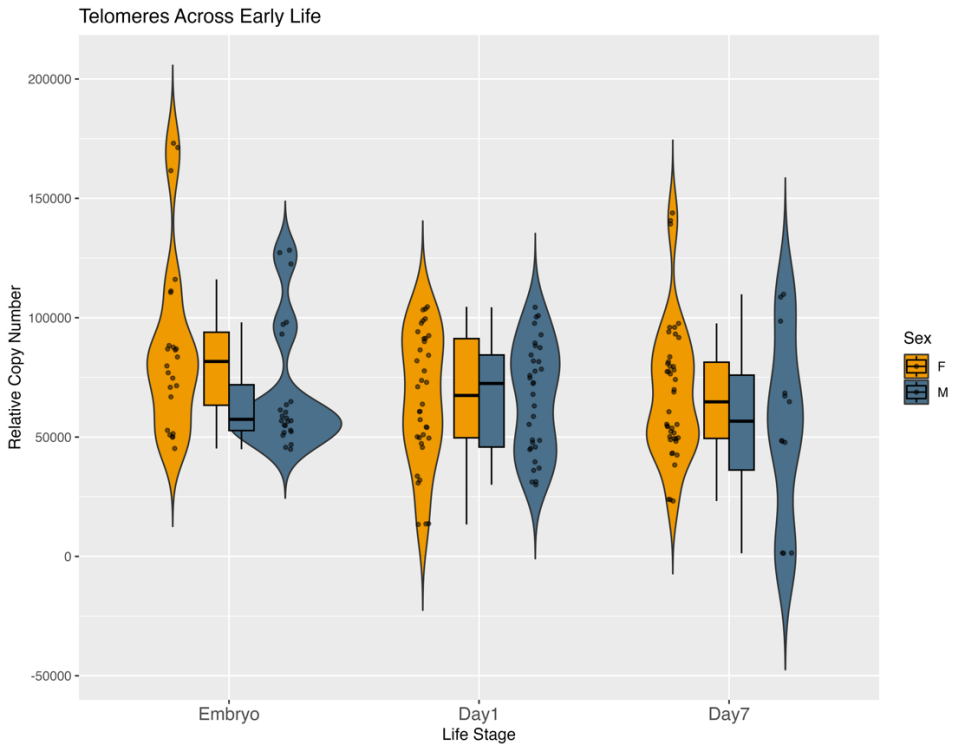

Figure 1.5 Relative Telomere lengths across experimental time points. Telomere repeats per autosomal genome was log transformed for data to be more normally distributed. Sex is shown with males (blue) and females (orange) separated. No significant differences were found across age group or across sex, or their interactions.

Discussion

In this study, we utilize the brown anole lizard (*Anolis sagrei*) to analyze early life telomere dynamics in a model reptile, test for sex-specific differences in telomere dynamics, as well as establish a baseline telomere length in reptiles. We focused on two hypotheses. Firstly, using our three age groups, we hypothesized that *A. sagrei* would show a telomere length decline from the embryonic treatment to the day 1 treatment followed by a slight increase in telomere length from day 1 treatment to the day 7 treatment if telomerase was subsequently activated. However, our results show that there was no significant difference in telomere lengths across these time points.

The non-significant differences in telomere lengths across the time points selected for this experiment are somewhat unexpected. When comparing the results to similar studies that looked at 7 days to 15 days of life in zebra finches, telomere attrition was present in the results (Salmon et al. 2021).

However, there are factors that may help to broaden the interpretation of our results from embryo to age Day 7. The lack of variation in telomere length across time points could potentially be explained by continuous telomerase expression. It may be that the enzyme telomerase is highly active during these early life points and would counteract any telomere shortening that may take place resulting in the nonsignificant difference in telomere lengths across observation points. A second interpretation is that the process of hatching and initial exercise outside of the egg doesn't induce cellular stress that results in telomere damage despite the expected new bursts of reactive oxygen species that would be expected. Additionally, studies in other reptile species have demonstrated significant maternal and paternal effects on hatchling telomere length that may explain a lot of the variation in our data (Olsson et al 2022). Since the parents of the animals used in this study were group housed, we can't test for maternal and paternal effects in these data.

A notable limitation of this study was that this was a cross-sectional study and not longitudinal, which would better allow us to take into account the innate variation among individuals due to

parental effects as seen in other lizards (Olsson et al 2022). With the size of the lizards at the desired timepoints, it would not be possible to collect adequate tissue without having to sacrifice the individual at this time point. Additionally, the total blood volume for *A. sagrei* at these time points is relatively low and require euthanization to obtain adequate volumes for quality DNA isolations as well as desired DNA concentrations.

Secondly, we hypothesized that there would be sex-specific differences in telomere length with female *A. sagrei* having longer telomeres. Because adult wild caught *A. sagrei* males have shorter telomere lengths than wild caught females, they can either diverge at some point in the development and aging process, or they could be different at hatching. To test this, we first had to develop, test, and validate a molecular sexing protocol by adapting methods from Ravotsos and Kratochvil 2016. Once optimized, this protocol was used to define the sex of the study embryos and hatchlings. When testing for sex-specific differences in telomere lengths we found no significant difference between sexes at these early life timepoints, indicating the sex-specific differences arise later in life. To further build on this research, it would be impactful to add more time points for observation to better understand at what age we begin to see the sex-specific differences in telomere lengths that are seen in wild caught adult male and female *A. sagrei*.

While acknowledging limitations, the results of this work illuminate that in *A. sagrei* blood cells telomeres are stable from an embryonic stage of development to one week of life, and that telomere dynamics are not different between male and female as this early life stage. To expand upon this work, if the experiment were to include the quantification of telomerase expression levels that would provide further insight on if telomere maintenance and repair is elevated in early life and thereby counteracting any telomere damage. Additionally, if the time points observed were expanded up to and past sexual maturity, this would hypothetically allow for the illumination of at what time point sexual dimorphism would impact telomere lengths. Lastly, looking across different tissues would allow for a better understanding of early life telomere dynamics overall. It has been shown in birds that blood telomere lengths are not always indicative of tissue telomere lengths depending on life stage (Schmidt et al. 2016). Additional studies in birds have found that telomere lengths vary between blood and feather samples

between hatchlings and adults with hatchlings having longer telomeres in blood and the adults showing no significant difference between tissues (Stier et al. 2020).

A major impact that this study provides is filling a gap in knowledge by illuminating early life telomere dynamics in reptiles and develop a baseline telomere length for a model reptile. Moreover, the importance of understanding telomere dynamics in reptiles is to develop a comparative model to understand what differences occur across reptiles, mammals, and birds in regulation of telomeres. Additionally, this study developed a molecular sexing validation method for reptiles to allow for sex specific comparisons at an embryonic level.

Data Accessibility

Experimental data has not yet been published but can be found at:

TS_Lab_ AnoleAging > NIH_R15_Anole_ModelAging_IGFs > Aim1_LabLongevity_Aim > Telomeres_EarlyLife_Ryan > Experiment1_AcrossHatching > Data_Analysis > Experiment_1_Experimental_Data

Chapter Two: Telomere length during early life growth and exposure to insulin-like growth factors in the brown anole lizard

This chapter will be included in a larger manuscript for submission:
Beatty, Hardin, et al, Graze, Schwartz. An experimental manipulation of IGF1 and IGF2 and its effects on sexually dimorphic growth rate and telomere dynamics.

Introduction:

In many species, males and females differentially invest in rates of growth and reproduction. In species where males have larger body size relative to females, males often invest more energy into accelerated growth in the early stages of their life to obtain the larger body size to establish territory more effectively or combat other males for optimal mating purposes. A faster growth rate can be achieved by a higher rate of cellular division that may reduce investment in cellular maintenance, potentially having a negative effect of lifespan (Barret and Richardson 2011). When looking at how organisms respond to early life growth, studies have found that while faster growth can provide advantages, faster growth can also have repercussions that may not be observed until later in life (Metcalfe and Monaghan 2001). Examples of this life strategy can be seen in zebra finch males experiencing greater mortality rates when having to catch-up in growth rate (Birkhead et al. 1999). Similarly, this can also be observed in mice where post-natal catch-up growth was associated with shorter telomeres in kidneys (Metcalfe and Monaghan 2001).

Telomeres, the caps at the ends of chromosomes, are one of the biomarkers of cellular stress and senescence, and telomere dynamics is also a point of high interest in sexually dimorphic species. Although there is a general trend of telomere attrition as individuals age, it is also common for there to be sex specific differences in attrition rate. This can be seen in humans and sea lions, both species with sexual size dimorphism with larger males that have shorter telomere lengths relative to females (Seifarth et al. 2012; Izzo et al. 2011). While in a long-lived scavenger avian species, the Andean condor, the female is larger body size, the female telomere length is shorter than that of the male (Gangoso et al. 2016). However, reptiles seem to have less predictable telomere dynamics. A study in Siamese cobras found there to be a negative correlation between

body size and telomere length in male cobras but not in females. Furthermore, females younger than five years had shorter telomere lengths than males, but longer telomere lengths after sexual maturation (Singchat et al. 2019). Lastly Ujvari and Madsen observed a different trend was in pythons showing that there is no significant decrease in telomere length from 1 year of age to 9 years of age. This was observed between male and female pythons, with sex specific differences being present showing females having longer telomere lengths (Ujvari and Madsen 2009).

One of the molecular networks that regulates the relationship between cellular and organismal growth and cellular maintenance is the insulin and insulin-like signaling network (IIS). The insulin-like growth factors, or IGFs, include IGF-I and IGF-II, that are evolutionarily conserved peptide hormones related to insulin. IGFs activate the IIS and play a pivotal role in the growth and development of all vertebrates studied (Denley et al. 2005; Wood et al. 2005). In humans, IGF-I is acknowledged for its critical roles in the development and functional maturation of the central nervous system, skeletal system, and reproductive organs (Woods et al. 1996). Examples of the role that IGFs play in mice are shown by various studies that compared IGF-I and IGF-II knockout mice to their litter mates, showing the birth weight of mice from both knockout treatments being 60% of the wild type (Duan et al. 2010). Additionally, over expression of IGF-I in mice resulted in an increase of body weight by 30% in comparison to wild type litter mates (Duan et al. 2010).

The function of the IIS network has been less studied in reptiles and birds (Schwartz & Bronikowski, 2016), but current studies suggest a similar role in promoting growth and reproduction. In garter snakes, strong correlation between levels of circulating IGF-I and fecundity has been observed in females, with larger clutch sizes having higher levels of IGF-I (Sparkman et al. 2009). In the same study, it was also found that IGF-1 levels increased as the body size increased in males and nonreproductive females (Sparkman et al. 2009). In a study in pied flycatchers, it was found that the introduction of exogenous IGF-I via subcutaneous injections during embryonic development resulted in increased growth rate, body size, and tarsus length by day 7 of observation. These findings were in comparison to control nestlings with the IGF-I injected group reaching fledging size sooner than the control group (Lodjak et al. 2017). Based on the findings of these previous studies in birds and ectothermic reptiles, it can be

hypothesized that the IGFs function similarly between mammals and other amniotes, such that experimentally increasing levels of IGF-I in reptiles would be predicted to increase growth rate during early life.

When observing the relationship between IGFs levels and sexual dimorphism across species, variation can be found across studies. A study in zebrafish found that when the IGF-I levels were reduced, both male and female zebrafish would show reduced growth in comparison to wild type but had sexually dimorphic metabolic effects where male zebrafish use IGF-I for cell cycle control compared to female zebrafish using it for glucose uptake (Zeng et al. 2022). In reptiles, a study using Madagascar ground geckos, where the species experiences sexual size dimorphism with larger males, observed spikes in IGF-I were found in males in comparison to females during the development of dimorphism leading up to sexual maturity. It should also be noted that IGF-I expression remained elevated after sexual maturity in males (Meter et al. 2022). Cox et al. (2022) revealed pronounced sexual dimorphism in the expression of the GH/IGF network among adult brown anole lizards *(Anolis sagrei)* where the male *A. sagrei* showed significantly higher expression levels of many upstream genes in the GH/IGF network that included both IGF-I and IGF-II. Furthermore, the study observed that juvenile male and female brown anoles exhibited similar expression levels of those same genes in the GH/IGF network in comparison the adult slender anoles (*Anolis apletophallus*) that do not experience sexual dimorphism (Cox et al. 2022). This study also found that adult *A. sagrei* also showed sex-biased expression levels on liver and skeletal muscle tissues in adults while the juveniles did not show this sex-biased expression levels when looking at the same tissue types (Cox et al. 2022). The previously mentioned studies primarily emphasize the associations involving IGF-I, leaving a knowledge gap regarding the potential causal roles of IGF-II in sexual dimorphism and its role in defining sexual dimorphism.

While the IIS has been heavily studied, between 1984 and 2020 most studies have focused on the function and manipulation of IGF-I with studies around IGF-II being less abundant and only accounting for 29.9% of all publications involving IGFs (Beatty et al. 2022). A commonly used mammalian model in IGF studies is mice, however, these studies have shown that IGF-I expression can be observed across all stages of life whereas IGF-II can only be observed during

the embryonic development of mice (Wolf et al. 1998). Prenatally, mammalian IGF-II is known as an imprinted gene where it is expressed paternally in the fetus and placenta (Constancia et al. 2002), but the effect of IGF-II expression has in juvenile and adult organisms is relatively understudied (Beatty et al 2022), However, IGF-II expression can be observed throughout the lifespan in humans (Sussenbach et al. 1993; Baral and Rotwein 2019) as well as other amniotes (Beatty et al. 2022; McGaugh et al. 2015, Rotwein 2018). With IGF-II not being expressed in all life stages in mice, this absence presents the need for another comparative model organism to be developed to better understand the role IGF-II may play in adulthood of humans and other organisms. To further illuminate the effects of both IGFs, using *A. sagrei* as an experimental model, due to their expression of IGFs at embryonic levels as well as adulthood, differences in adult male and female body sizes, and differences in males and female lifespan to better understand the role IGFs may have across life would prove useful (Beatty et al. 2022).

Here we use the brown anole lizard (*A. sagrei*) to experimentally test the relationships between IGF injections, sexual dimorphism in growth rate during early juvenile development, and telomere dynamics. In this experiment, we are attempting to manipulate growth rates with injections of insulin growth factors I and II (IGF-I and IGF-II). I hypothesize that brown anoles will exhibit sex-specific divergence in overall telomere length in the early stages of developing sexual dimorphism (~ 3 months) and this will be reflected in relatively shorter telomeres in males than females. Secondly, I hypothesize that injection of the growth hormones, IGF-I or IGF-II will result in shorter telomeres relative to vehicle injections, with sex specific effects. To address these hypotheses, we experimentally inject recombinant brown anole IGF-I or IGF-II proteins into juvenile brown anole lizards and measure telomere length in red blood cells at Week 1 and Week 13 of development.

Methods:

Egg collection

In the summer of 2020, eggs were collected from the Schwartz Lab breeding colony at Auburn University (IACUC PRN: 2019-3521). The eggs were incubated in petri dishes (VWR International, 60x15mm, Cat # 25384-090) that contained 1 tbsp of 50:50 mass ratio

water/vermiculite. Each egg was assigned an "AA" number that was recorded for identification throughout experimental processes. The petri dish was labeled with the "AA" number printed on a sticker (Avery Multiuse Removable Round Labels, Product #6450) along with the egg collection date to predict the hatch dates of individuals. The Petri dishes were wrapped in 2" strips of parafilm (Bemis Parafilm "M" PM-999/ 4inx250ft roll) to prevent desiccation during incubation, and later wrapped twice with 2" strips of parafilm due to moisture loss from cracks developing in the single layer of parafilm.

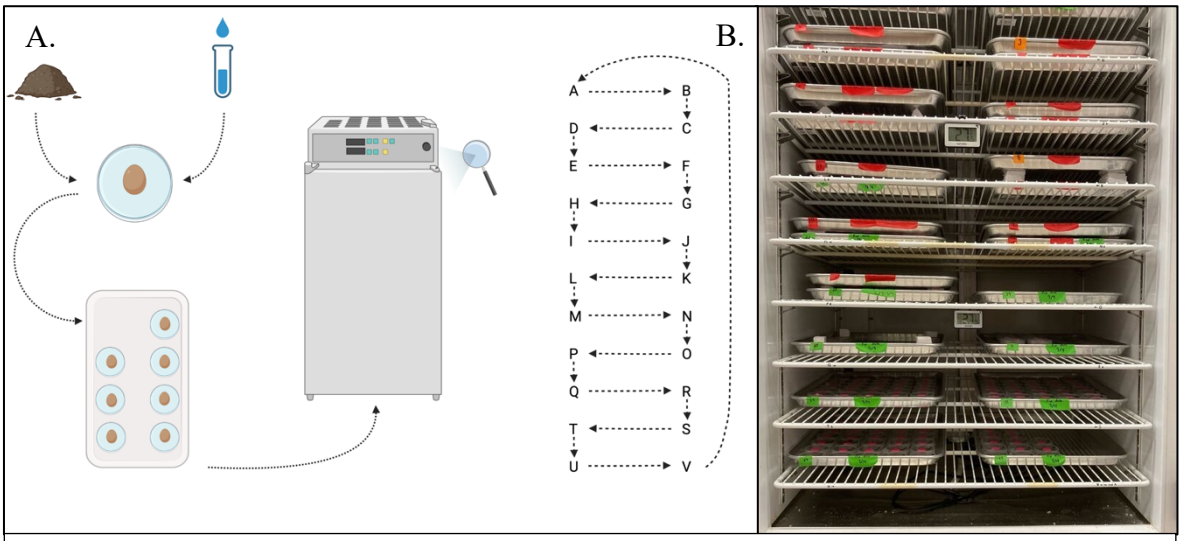

Figure 2.1. Egg processing and incubation rotation system.
A. Diagram showing how each egg was processed and placed on a baking tray before being placed into the incubator.
B. Representation of the rotation schedule in order for each egg to experience the same temperatures throughout the incubation process up to hatching unless designated to the "embryo" treatment.

Once egg processing was complete, the eggs were placed in an incubator (Percival, I36NL) at 27°C. The incubator had 11 shelves total with room for two metal trays side by side (Baker's Mark, half size perforated pan, measurement "0.054 M3") making the total number of tray positions 22. When all 22 positions were occupied by trays of eggs, trays would be stacked two high with small squares of 2" x 2" foam placed in each corner between the petri dish and the tray above them to prevent petri dishes from sticking to the tray placed on top. The eggs from the most recent collections would be stored underneath previously collected eggs to prevent missing

a hatchling during the routine checks for new hatchlings. Each rack had two positions indicated by letters. This was to ensure that each egg would receive the same temperature exposure by rotating the trays through the incubator, changing one position every Tuesday, Thursday, and Saturday.

Treatment Groups.

Eggs were randomly assigned upon hatching to two age groups that determined when the individuals would be euthanized: (1) Week 1, hatchlings euthanized at 6-7 days of age, prior to any injection treatments; (2) Week 13, mid-juvenile stage, after having received injections at age 1 week and age 7 weeks of age. These Week 13 individuals were assigned one of three injection treatment groups: IGF1, IGF2, or vehicle (describe in more detail below). These treatments were double-blind to the persons doing the injections, collecting measurements, and the statistical analyses, and they were coded as: “Yellow”, “Pink”, and “Orange” (not respectively).

Housing and Feeding

Immediately following hatching, each animal was assigned a unique toe clip and experimental ID (AAXX) and placed in an individual juvenile enclosure. Juvenile enclosures consisted of 5.5”x7” tubular mesh enclosures (size) containing a 5” reptile cage carpet, one 6” bamboo rod, two artificial maple leaves, and one 9” ivy vine. Hatchlings were house on metal racks under UV-B bulbs (Product) on a 14:10 hour light-dark cycle. All lights in this region were on consistent schedules by the time we had hatchlings. Upon hatching, individuals were placed on a “Neonatal” rack labeled by weekday. For their first week of life, the hatchlings remain on this shelf for ease of location for injection on Day 6/7. Following their first injection, the hatchlings were moved to a secondary rack through their second and third injection (when we switched to adult cages). Hatchlings were rotated through all 6 shelf locations on their designated racks, moving one shelf per feeding event to reduce the risk of location effects.

Diet was age dependent (Table 2.1) and consisted of a mixture of calcium gut loaded *Gryllodes sigillatus* crickets and *Shelfordella lateralis* Cockroaches that were dusted with Herptivite supplement powder (Product #) and calcium powder supplement (Product #) at each feeding. Lizards remained on a 14:10 hour light:dark cycle under one UV-B bulb (product #)

for the duration of the experiment and were misted 1-2 times daily with water. Individuals remained in these cages for the duration of the experiment.

In May of 2020, the COVID 19 pandemic caused a cricket shortage in the United States. This led to our typical supplier (Georgia Crickets) to be unable to supply us with our usual orders of American House crickets. At that point we switched to TopHat Crickets, which had the *Gryllodes sigillatus* species in stock. From that point forward, we continued to order the *G. sigillatus* (tropical house cricket) from TopHat. There were issues periodically with food being delivered late (ex. Northeastern storm in March 2021) or with no live crickets. In those cases, a second overnight order was placed, and animals in highest priority were supplemented with either fruit flies or cockroaches depending on age and food availability.

Table 2.1: Feeding schedule for animals in this study

Age	**Monday**	**Wednesday**	**Friday**	**Sunday**
Neonate	5-7 Fruit Flies	5-7 Fruit Flies	5-7 Fruit Flies	5-7 Fruit Flies
1 Wk	7-10 Fruit Flies	7-10 Fruit Flies	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies
2 Wk	7-10 Fruit Flies	7-10 Fruit Flies	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies
3 Wk	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies
4 Wk	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies 2 Nymph Cockroaches	7-10 Fruit Flies
5Wk	5-7 Fruit Flies	5-7 Fruit Flies	5-7 Fruit Flies	5-7 Fruit Flies

	2 Nymph Cockroaches	2 Nymph Cockroaches	2 Nymph Cockroaches 1 ten-day old Cricket	
6 Wk	5-7 Fruit Flies 1 Nymph Cockroaches 2 ten-day old Crickets	5-7 Fruit Flies 1 Nymph Cockroaches 2 ten-day old Crickets	5-7 Fruit Flies 1 Nymph Cockroaches 2 ten-day old Crickets	5-7 Fruit Flies
7 Wk	5-7 Fruit Flies 1 Nymph Cockroaches 2 ten-day old Crickets	5-7 Fruit Flies 1 Nymph Cockroaches 2 ten-day old Crickets	5-7 Fruit Flies 1 Nymph Cockroaches 2 ten-day old Crickets	
8-10 Wk	3 ten-day old Crickets	3 ten-day old Crickets	4 ten-day old Crickets	
11-13 Wk	2 small (½") crickets	5 ten-day old Crickets	2 small (½") Crickets	

Body size and growth rate

Within 24 hours of emerging from the egg, hatchlings were collected from the incubator and processed. Processing included assigning each individual a toe clip for identification purposes, and recording snout vent length (SVL), mass, sex and back pattern. Sex was initially identified through the presence (males) or lack of (females) post-anal scales and by back pattern (yellow pattern in females) when possible. Mass was measured to the nearest 0.001g, and SVL was measured using a ruler to the nearest 0.5mm. Following processing each animal was placed in an individual juvenile enclosure labeled with the unique experimental ID and toe clip. Morphological measures continued every six weeks for a period of six months, when we expect individuals to become sexually dimorphic. At Week 7, mass and SVL were collected immediately preceding injection. Additionally, sex was again visually verified at Week 13.

Injections

Injection Media Preparation:

Gelatin injection media (24% w/v) was prepared by dissolving 24g of sterile gelatin (VWR; 97062-618) in 100mL of DEPC treated water in a 500mL screw cap jar. Mixture was autoclaved in InstantPot on high pressure for 20 minutes. Quick release pressure value was used immediately following the pressure cycle, and the "keep warm" setting was used to maintain pipettable viscosity until gelatin was aliquoted into 2mL tubes at a volume of 100μL. Gelatin aliquots were stored at 4 C for the duration of the experiment.

IGF1 and IGF2 protein (KingFisher; RP1675A and RP1676A) was resuspended in sterile water to a concentration of 1μg/μL. Protein is prepared at the beginning of each round of injections (every 6 weeks). The exact dose of protein is dependent on the average mass of animals at the time of injection. At week 1 we used XX dilution, at Week 7 injection we used XX dilution. Sterile 0.9% saline (BioVision; 75837-682) was used for control injections in place of IGF protein. Diluted proteins/saline were aliquoted into 103μL portions to eliminate repeated freeze-thaw cycles.

Injection Protocol:

At the first injection timepoint (Week 1), a researcher independent of the injection procedures randomize the treatment to a designated color. IGF1, IGF2, and Vehicle (saline) were randomly assigned to either yellow, orange, or pink. All protein labels were replaced with color codes to ensure injectors were blind to treatments for the duration of the experiment. To avoid cross-contamination, an independent microsyringe was color matched to each treatment.

Animals were randomly assigned to IGF treatment groups of Vehicle, IGF1, or IGF2 injections with corresponding colors of yellow, pink, and orange for treatment identification. Throughout summer and fall of 2020 injections were conducted at 1 week (age day 6 or day 7) and 7 weeks of age. Two injectors were present daily. Each treatment was split evenly between the two researchers, randomizing the order of treatments to be injected daily.

Immediately before injections, properly diluted protein aliquot is mixed 1:1 with 24% gelatin for a final injection dose of 5µg/gram body mass injection in 12% gelatin. Protein was added to the 2mL tube containing 24% gelatin and the color-coded treatment sticker was transferred to the 2mL tube. The tube was dated, warmed to 40 degrees C, and shaken at 250rpm until thoroughly mixed.

A 250µL syringe was filled with each injection media, capped with a disposable 27 gauge ½ inch subcutaneous injection needle, and fitted with an auto dispenser to regulate 5µL injection volumes. Syringes were kept on a heating pad while in use to avoid hardening of gelatin in needle.

Animals were given a subcutaneous injection above the front right shoulder blade. In the case that the injection in that location fails, a second attempt was made over the left front shoulder blade. Any notes on secondary injections, possible leakage, or other complications were recorded on the injection data sheet. Any remaining protein was placed back in the proper tube and kept at 4 degrees C for the next injection. No media was kept for more than 1 week at 4 degrees C.

Animals were immediately placed back in their enclosure for recovery.

Dissections

A set of 25 individuals were euthanized and dissected at age Week 1 prior to any Treatment Injections; these will be referred to as Week 1 Age Class. A second set of 25 individuals that received each type of injections at Week 1, Week 7 and were dissected at age Week 13; these will be referred to as the Week 13 Age Class.

Prior to dissections, 1.5ml screw cap tubes (Thermo Scientific Screw Cap Micro Tubes, 1.5ml, Ref# 3474) would be labeled with the "AA" ID as well as an abbreviation that corresponds to the tissue type that is stored in it before snap freezing. The tissue types collected for embryonic, within 24 hours of hatching, one week, and 3-month time points were blood (BL), brain(B), heart(H), liver(L), muscle(M), and body (BODY). Equipment used in dissections included dissection boards (Simport, M630-1 DsipoCut Dissecting Board, 152 x 203mm), dissecting pins (Dritz Home 214500 Bulk Package of Nickel-Plated Steel T-Pins, 350-Pack, Silver), scalpel, fine

dissection scissors, forceps, butcher scissors for euthanization, and a jeweler's scale (for three month time point only). Before dissections began and in between each individual dissection, all tools were disinfected by rinsing them in DI water, followed by 10/1 DI water/bleach mixture, rinsing in DI water, RNAse away, and a final rinse in DI water. For all time points the anoles were placed dorsal side down on a transparent ruler in order to measure the snout/vent length in mm. After the SVL was recorded, the lizard would be placed dorsal side down on the middle finger with the thumb and pointer finger holding it is place. Once the lizard was secured, the butcher scissors were positioned at the base of the skull and quickly used to decapitate the lizard followed by pithing. Once decapitated, capillary tubes (Kimble 41B2501, Micro-Hematocrit Capillary Tubes, Heparinized, Red Coded Pack of 200) were used to collect the blood tissue from the head and neck wounds. This blood volume would then be estimated in µl and then ejected into a screw cap tube using a capillary plunger. The blood tissue was the only tissue collected that would be placed in DNA/RNA shield for storage with the 3-month time point blood tissue being snap frozen. The anole was then lain dorsal side down on the dissection board and dissection pin was placed distally on each limb. Fine dissection scissors were then used to cut a vertical line from the neck wound down the midline of the dorsal side to the pelvic area. The heart tissue would be removed using fine dissection scissors, placed in a screw cap tube, and snap frozen. The liver tissue would be removed and before placing the tissue in the screw cap tube and snap freezing the tissue, the gallbladder would be removed to prevent tissue contamination. For muscle tissue, the right hind leg would then be removed from the hip. The skin would be removed starting at the knee in a proximal direction in order to expose the thigh muscle tissue. The thigh would be positioned so that both the hamstring and quadricep area would be taken for tissue sampling. Using a scalpel, muscle tissue would be dissected away, placed in a screw cap tube, and snap frozen. Once all individual tissues were dissected and properly snap frozen, the body of the anole would be placed in a screw cap tube and snap frozen as well. After all tissues were collected and snap frozen, the timer would be stopped in order to get a total dissection time for all tissues. Once all dissections were complete and all tissues were snap frozen, they were stored in a -80 until they are needed for analysis.

The dissections for the 13 Week time point varied in that all tissues were weighed using a jeweler's scale with the addition of two tissues being collected per dissection, fat tissue and

gonad tissue. This dissection process used the same dissection tools with the addition of a weigh boat (VWR Disposable Square Weighing Boats, 46x46x8mm, 10803-136) to place tissues during weight measurement to get the mass of the tissues collected before storing them in respective screw cap tubes for snap-freezing. The weigh boat was divided into six quadrates using the permanent marker with the labels being “B” on the top left quadrate for brain tissue, “H” on the top right quadrate for heart tissue, “L” on the middle right quadrate for liver tissue, “M” on the bottom right quadrate for muscle tissue, “G” on the bottom left quadrate for gonad tissue, “F” on the middle left quadrate for fat tissue. The reason or sectioning the weigh boats is to prevent contamination of tissues.

At week 1 (before injections) 25 individuals were euthanized, dissected, and blood was collected for telomere analyses. At Week 13, a subset of animals from each injection treatment group were dissected and blood was collected for telomere analyses: 25 yellow, 21 pink, and 24 orange samples were used for further experimentation (Table 2.2 [summary of sample sizes and sexes). Comparisons across these time points and samples will allow me to evaluate the major life history stress of early life growth with IGF treatments and the effects these variables have on telomere length as a measure of stress.

DNA isolations

In preparation for DNA isolations, all samples were randomized in order of isolation to prevent biases across day of DNA isolation affecting experiment groups. I conducted DNA isolations from 5ul – 10ul of whole blood (total volume varied depending on the individual) using a Gentra Puregene DNA Isolation Kit A (Qiagen), using the “Cultured Cells” protocol provided by the manufacture. Gel electrophoresis was conducted to assess DNA quality by loading 5 ul of DNA on a 1% agarose gel (0.5gm agarose in 50 ml TAE) running at 100V for 1 hour. DNA was quantified using Thermo Scientific NanoDrop 2000 Spectrophotometer. A dilution of 5 ng/µl was made for every sample to standardize the concentration of DNA for the telomere analysis and sex validation.

Molecular Sexing via PCR.

To validate the sex of the individuals to make comparisons between male and females, the molecular sexing protocol from Chapter 1 was also used here to validate the sex of *A. sagrei* samples. *A. sagrei* has XX/XY sex determination, a multiplex qPCR protocol was developed to quantify copy number for an X-linked gene (atp2a2) relative to an autosomal gene (adarb2) based on the methods from Ravotsos and Kratochvil (2016).

Primer sets to amplify the genes atp2a2, adarb2, tmem132d, and gapdh, along with a probe were redesigned to complement the *A. sagrei* genome (Geneva et al. 2022) and validated via gel electrophoresis after PCR to ensure each set of primers produced the expected PCR product size (Table 1.2). After validating the primer sets amplified the correct size target, complementary qPCR hydrolysis probes were ordered (Integrated DNA Technologies) for each primer set and tested via qPCR and high PCR efficiency was confirmed based on the Absolute Standard Curve.

The multiplex qPCR was run in triplicate in 96-well white-walled plates (BioRad cat. no. HSP9655), with BioRad Microseal 'B' seals (cat. no. MSB1001). The 20ul volume reaction consisting of 10ul (1X) of IDT PrimeTime Gene Expression 2x Master Mix, 0.4ul (final concentration XXX) of each Adarb2 and Atp2a2 primers and probe (all at working concentration of 10 μM), 5.6ul of molecular grade water, and 2ul of 5ng/ul of DNA template per reaction. The following thermal cycle conditions were used on a BioRad CFX96 Optics Module qPCR machine using the Cy5 channel to detect the fluorescence of Adarb2 and the Hex channel to detect the fluorescence of Atp2a2. Thermal Cycle was as follows. Stage 1: a single cycle of 95°C for 3:00 min; Stage 2: two cycles of 95°C for 0.15s, 49°C for 0.30s, 55°C for 0.30s; Stage 3: thirty five cycles of 95°C for 0.15s, 56°C for 0.30s, 60°C for 0.30s, plate read; Stage 4: melt curve 60°C to 95°C increment 0.5°C 0.05s plate read. Note: Stage 2 and Stage 4 are necessary for the Telomere amplification, but not for this molecular sexing. This protocol works equally well for molecular sexing if you delete these stages. The absolute standard curve was also run in triplicate on every plate and was used to calculate the starting quantity of target genes.

The primary objective of this protocol was to enabled testing for the X-linked gene copy number in relation to the autosomal gene copy number. QPCR was conducted on a training set of known

males (N=12) and known females (N=12) and the starting quantity of the X-linked gene was divided by the starting quantity of the autosomal gene. These X-linked gene/autosomal gene ratios from the training set produced distinct ranges for each sex resulting in a male X/A range of 0.45-0.65 and a female X/A range of 0.85-1.05. We validated these ranges for defining sex using a test set of a different known males (N=12) and known females (N=12). This protocol would allow us to validate the sex of all treatment groups to test for sex specific differences in telomere lengths across treatments. The sex of the experimental individuals was classified based on the ranges defined by these known males and females (Figure 2.2). Complete sample sizes for this study, including sex are in Table 2.2.

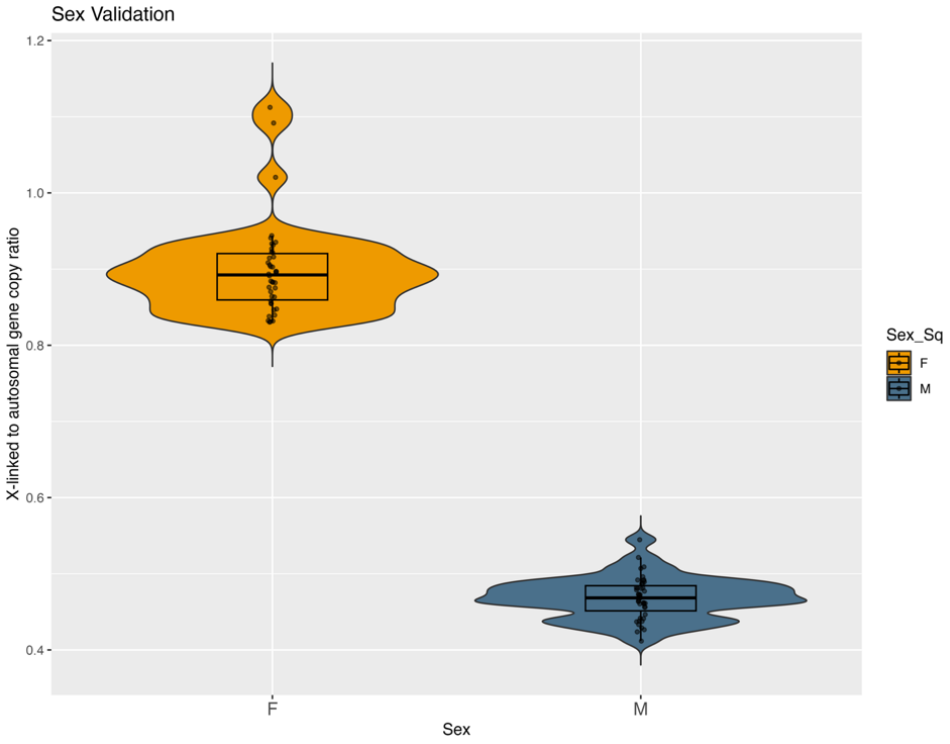

Figure 2.2 Molecular Sexing Results for This Study.

The Y-axis is the ratio of the starting copy number of the x-linked gene (Atp2a2) relative to the starting copy number of the autosomal gene (Adarb2). The ratio determined by dividing the sq of the x-linked gene by the autosomal gene resulted in two distinct groups for sex. The male brown anoles (M) will have one-half as many x-linked gene copies due to being XY and females (F) being XX. Using these two distinct groups, sex was able to be validated to test for sex specific differences throughout the experiment.

Table 2.2 Sample sizes separated by sex.

Age	Treatment	Male	Female
Week 1	**N/A**	**11**	**11**
Week 13	**All**	**32**	**29**
	Pink	**10**	**9**
	Orange	**12**	**9**
	Yellow	**10**	**11**
Total		**43**	**40**

Telomere Quantification via qPCR

The collection of the telomere length data followed the same protocol as described in Chapter 1. Experiment 2 standard curve was constructed in the same manner as experiment one with the addition of plasmid spiked H2O. To buffer against the bias caused by nucleotides binding to the walls of the tubes, which has a greater effect on the end of the dilution series, molecular grade water spiked to a final concentration of 2ng/ul PBR322 Vector (Cat # N3033S) and this Vector-spiked water was used for making the standard curve dilution series. The process of performing qPCR analysis (Bio-Ras CFX Real-Time PCR Systems) for this experiment was split into two separate qPCR reactions per individual. The first qPCR reaction was a multiplex for the sexing validation protocol with the complementary telomere qPCR reaction being ran on the same day. A total of eight qPCR reactions were ran within four days to gather all the data needed for analysis. Before doing any statistical analysis, the threshold line was adjusted on all qPCR data files to ensure a consistent comparison across plates for accurate data. Using absolute standards developed using a Gblock (described in Chapter 1) it was possible to quantify telomere lengths.

Statistical Analyses.

All statistical analysis were conducted in R using packages tidyverse, nlme, lme4, lmtest, bestNormalize, MuMin, emmeans, ggplot2, ggpubr, and devtools. The code is attached as an appendix. To test if the qPCR the reference gene was normally distributed and to log transformation to make it normal, we used the bestNormalize package (Peterson 2020). After

normalization, the reference gene was then tested to see if it differed between treatments, sex, and age respectively. We then tested for normal distribution of the experimental value of telomere repeats per reference gene and normalized those values via log transformation.

Does age class and sex affect telomere length?

To test for an effect of age class (Week 1 vs Week 13) or sex, or their interaction on telomere length, I conducted a linear model with the lme function in the lme4 R package.

Does sex and IGF injection affect telomere length at 13 Weeks

Since the animals euthanized at Week 1 did not have Treatment Injections, the Week 13 Age Class were subset to test for effect of IGF Injection Treatments or Sex, or their interaction on telomere lengths using a linear model with the lme function in the lme4 R package.

Does IGF injection, growth rate, and sex affect telomere length between 1 and 13 Weeks

To test for an interaction between IGF Injection Treatment, growth rate, and sex on telomere length we developed a three way interaction model using the lme function in the lme4 package. In this model, growth rate was the change in either mass or the change in SVL between Week 1 and Week 13 for each individual that was euthanized at Week 13.

Results

When testing for differences in telomere length across age and sex we found no significant differences between sexes with males having a trend of longer telomeres than females across both ages, but this was not statistically significant between sexes (p-value = 0.145). When testing for the individual effect of age, we found no significant difference in telomere length (p-value = 0.805) (Table 2.3)

Table 2.3 Individual effects of age and sex on telomere length

Statistical Test Group	Comparison	Std. Error	P-value
Age x Sex			0.501
Week_13	Week_1	0.052	0.805
Sex_Sq_Male	Sex_Sq_Female	0.047	0.145

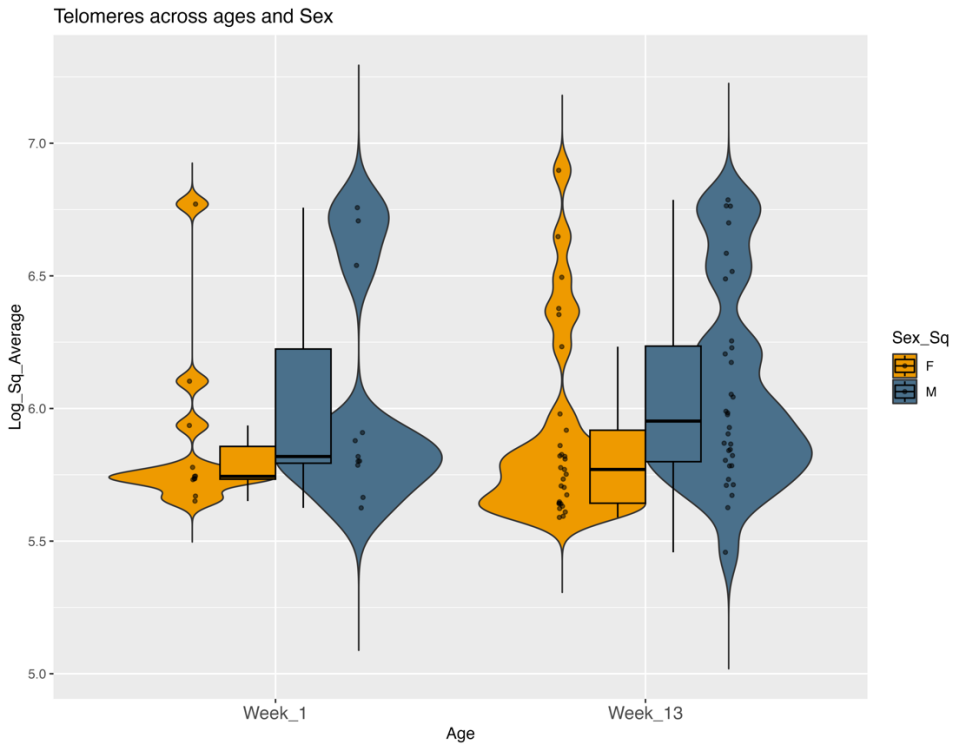

Figure 2.3 Relative Telomere lengths at week 1 and week 13 by sex. Telomere repeats per autosomal genome was log transformed for data to be more normally distributed. Each age group on the X axis with males (blue) and females (orange) separated. No significant differences were found across age or across sex, or their interaction.

Does sex and IGFs affect telomeres at 13 Weeks of Age

When testing for differences in telomere lengths across IGF1, IGF2, and vehicle injections and their interaction with sex, we found no significant interaction (p=0.187) across injection treatments. When looking at sex, males showed longer telomeres than females, but this was not statistically significant (p-value = 0.085). When looking at treatment comparisons we found no significant difference in telomere length between ("pink" vs "orange", p-value = 0.1535; "yellow" vs "orange", p-value = 0.5361) (Table 2.4).

Table 2.4 Summary of results testing for the effects of treatment and sex on telomere repeats at week 13.

Statistical Test Group	Comparison	Std. Error	P-value
IGF_Treatment			0.328
Sex			0.086
IGF_Treatment x Sex			0.187
Pink_Injection	Orange_Injection	0.0579	0.153
Yellow_Injection	Orange_Injection	0.0581	0.536

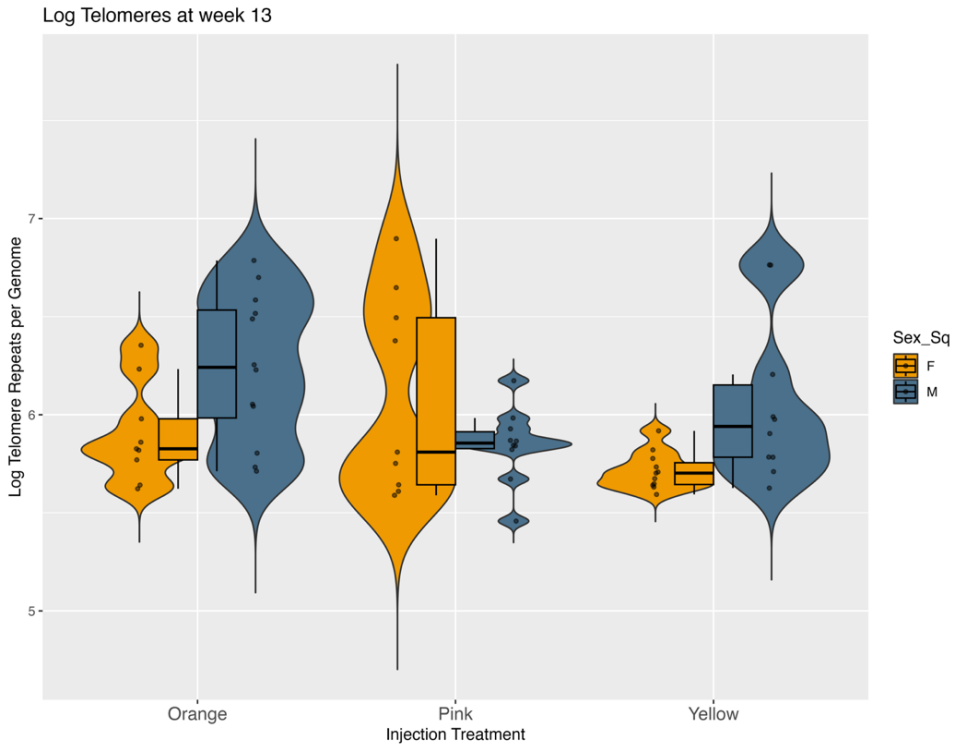

Figure 2.4 Relative Telomere lengths at Week 13 for each treatment by sex. Telomere repeats per autosomal genome was log transformed for data to be more normally distributed. Each injection treatments with males (blue) and females (orange) separated. No significant differences were found across injection treatments or across sexes, or their interactions.

Does IGF injection, growth rate, and sex affect telomere length between 1 and 13 Weeks?

In testing for the three way interaction of growth rate (either change in SVL or change in mass), sex, and IGF treatment on the telomere lengths we found no significant interaction (p-value = 0.596, Table 2.5). When removing the three-way interaction, the two-way interactions between growth rate and treatment were significant (p-value =0.03 and 0.001 for SVL and mass respectively, Table 2.5, Figures 2.5 and 2.6). When looking at telomere length change in the interaction between SVL and treatment we found telomere lengths would decrease with grown in both orange and yellow treatments (relative to Pink treatment). Additionally, there was a significant difference among sexes with males having longer telomers overall (p-value =0.056 and 0.037 for SVL and mass respectively, Table 2.5, Figures 2.5 and 2.6), although additional analyses are warranted to further explore this pattern in each treatment.

Table 2.5 Summary of results testing for the interaction of Treatment, growth rate (either change in SVL or change in body mass) and sex on telomere length.

Statistical Test Group	Comparison	Std. Error	P-value
SVL_Change x Treatment x Sex			0.596
SVL_Change x Treatment			0.003
Sex_SqM	Sex_SqF	0.044	0.056
SVL_Change x TreatmentOrange	TreatmentPink	0.031	0.002
SVL_Change x TreatmentYellow	TreatmentPink	0.029	0.015
Mass_Change x Treatment x Sex			0.286
Mass_Change x Treatment			0.001
Sex_SqM	Sex_SqF	0.043	0.037
Mass_Change x TreatmentOrange	TreatmentPink	0.464	0.001
Mass_Change x TreatmentYellow	TreatmentPink	0.49	0.002

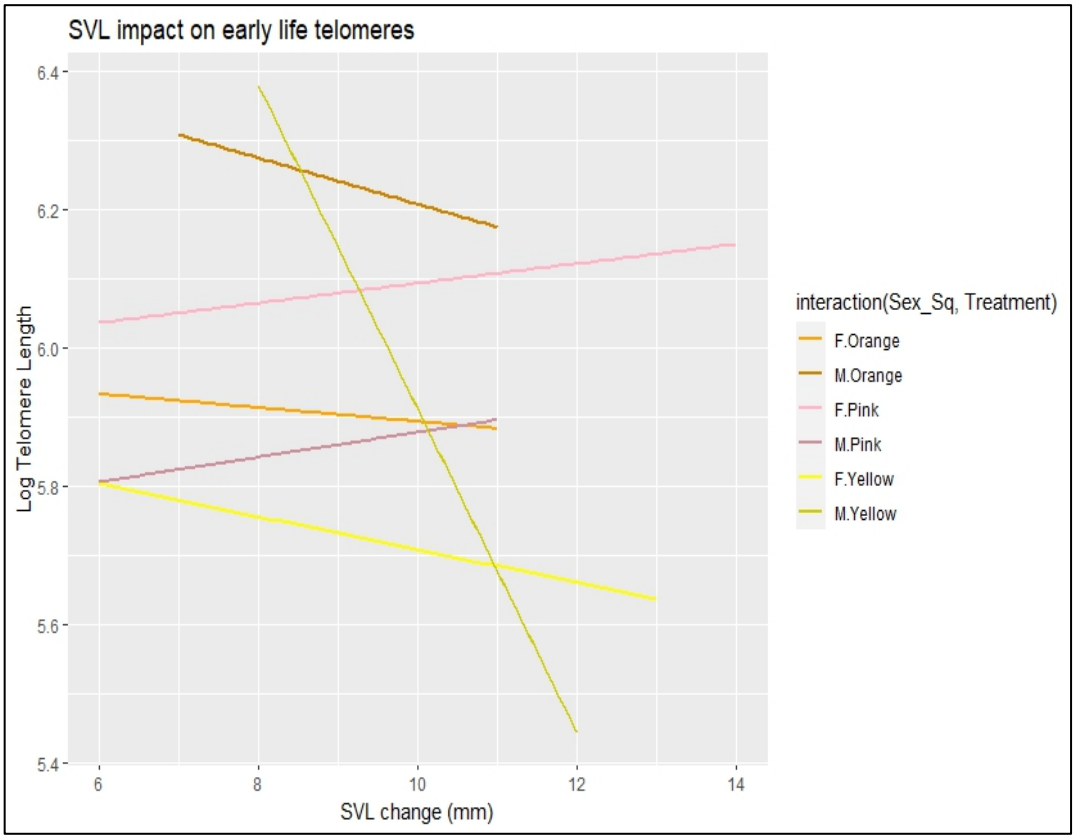

Figure 2.5 The interaction between change in SVL and IGF injections on telomere length at age Week 13. Pattern of results from the linear mixed effects models to test for relationships

between change in SVL and treatment (shown with corresponding color). Sexes are separated in this graph with males (dark hue) and females (light hue).

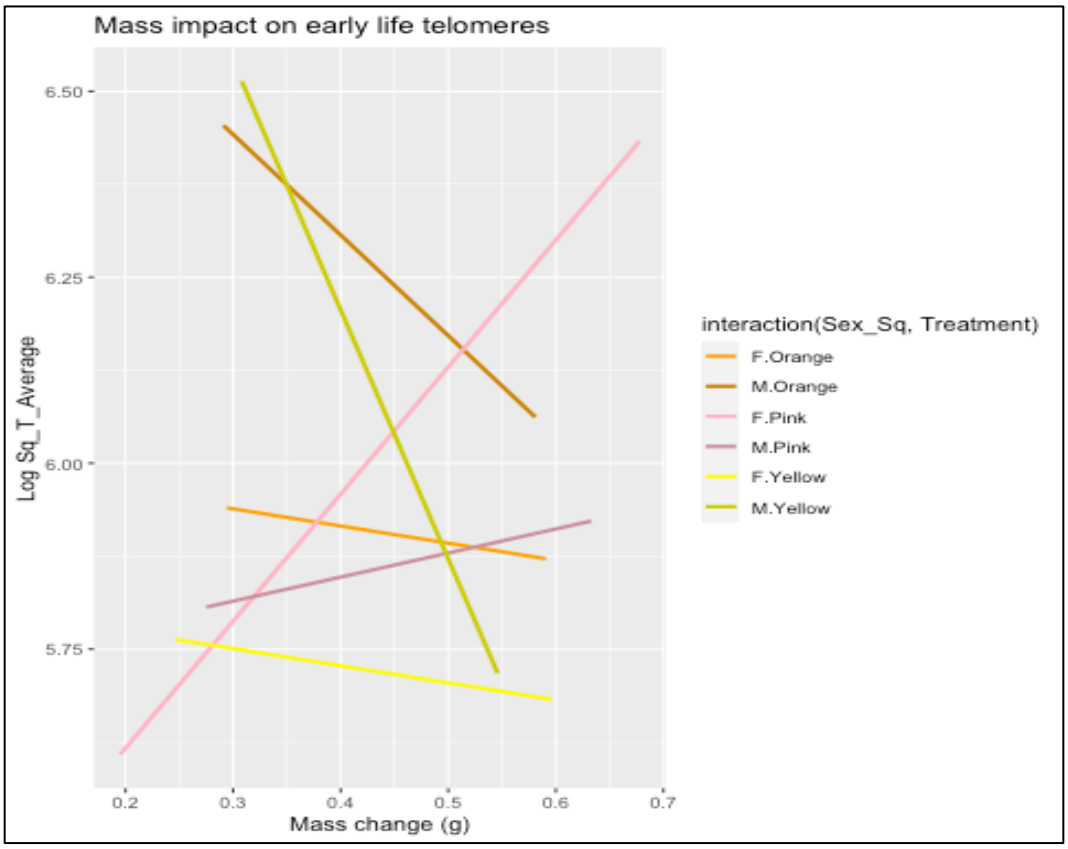

Figure 2.6 The interaction between change in body mass and IGF injections on telomere length at age Week 13. Pattern of results from the linear mixed effects models to test for relationships between change in body mass and treatment (shown with corresponding color). Sexes are graphed separately with males (dark hue) and females (light hue).

Discussion

In this study, we utilized the brown anole lizard (*Anolis sagrei*) to investigate the potential relationship between IGF injections, and development of sexual size dimorphism during juvenile development, and telomere dynamics. In addition to *A. sagrei* being sexually dimorphic, ongoing research has shown that wild caught, sexually mature male *A. sagrei* have shorter telomere lengths than their female counterpart at old ages (Reedy, unpublished). These findings suggest that there is a specific point where this telomere length divergence takes place. To further

examine the influence of the growth factors, IGF-I and IGF-II, on *A. sagrei*, we focus on two hypotheses. Firstly, we hypothesized that brown anoles would exhibit sex-specific divergence in overall telomere length as age class got closer to showing sexual dimorphism, leading to relatively shorter telomeres in males compared to females. We measured telomere length in red blood cells at two time points: Week 1 and Week 13 of development. Our results show that there is not a significant change in telomere lengths within the first 13 weeks of life between male or female *A. sagrei*. These findings suggest that telomere length divergence occurs at a later life point leading up to the sexual maturation of *A. sagrei.* This is in contrast to the findings by Ujvari 2009 suggest that in pythons there is a significant increase in the telomere lengths of individuals within juveniles within their first year of life. These findings would also contradict findings in birds that showed between day 3 and day 20 common tern chicks lost 7.5 telomeric pairs (Vedder et al. 2017).

Secondly, we hypothesized that the injection of growth hormones IGF-I or IGF-II, would lead to shorter telomeres relative to control (vehicle) injections, with potential sex-specific effect, potentially via its effect on increasing growth rate. If true, manipulating growth factors could influence telomere length, potentially accelerating the attrition process. To address these hypotheses, we conducted an experimental injections of recombinant brown anole IGF-I or IGF-II proteins into juvenile brown anole lizards at week 1 week, and week 7 ages. We measured telomere length in red blood cells at Week 13 of development. We found a significant interaction between growth rate (both change in SVL and change in mass) and IGF treatment on telomeres. While the injection treatments are still color-coded, these findings suggest that at least one of the IGF treatments was having a physiological effect on telomeres, possibly through their effect on growth rate.

With one goal in this study being able to test for sex specific telomere length differences leading into sexual dimorphism, our results suggest that increased growth leads to shorter telomere lengths in some IGF treatments within 13 weeks with differences between sexes being observed. While these growth rates have been manipulated through IGF treatments, the results from this study suggest that increased IGF-I and IGF-II levels and growth do significantly impact telomere dynamics in reptiles within the first three months of life.

Additionally, we found a separate independent effect on of sex on telomeres with specific differences present when looking at growth rate and IGF treatments. Interestingly the pattern was opposite of what was expected, with preliminary data shows that *A. sagrei* are sexually dimorphic and show sexually mature males have significantly shorter telomere lengths than females in the wild (Reedy, unpublished). When looking at differences in sex we show that males have longer telomere lengths than females in two of the injection treatments. We also found the males telomere attrition rate seems to be more drastic by the week 13 time point in the two injection treatments where males had longer starting telomere lengths. This would suggest that at least one of the IGF treatments would also have sex specific differences on telomeres when observing growth rate. Future analyses will explore these relationships further as the animals age.

Major impacts of this work is that it provides the scientific community with multiple key findings on the early life telomere dynamics of *A. sagrei*. Firstly, by using the absolute standards in qPCR, this work will provide the baseline telomere length for a model species to facilitate cross species comparisons in early life telomere dynamics. Secondly, these results suggest that male and female brown anoles do differ in telomere lengths in early life from Week 1 to Week 13 (mid-juvenile stages) in the presence of IGF-I and IGF-II. With telomere analysis in reptiles being a relatively new area of research, these results can also provide a comparative model for telomere analysis in early life across species.

To further build upon this research, it would be impactful to understand at what point do we begin to see those telomere length differences between sexes that have not had growth manipulation. Seeing that these time points are not close to the sexual maturation of the model organism, it is not surprising that we observed no significant difference between male and female telomere lengths from Week 1 to week 13 of life. However, these findings do fill a gap in knowledge by showing this telomere length divergence has not yet occurred at these times.

A notable limitation of this study was that this was a cross sectional study and not longitudinal. With the size of the lizards at the desired timepoints, it would not be possible to collect adequate

tissue without having to sacrifice the individual at this time point. Additionally, the total blood volume for *A. sagrei* at these time points is relatively low and require euthanization to obtain adequate volumes for quality DNA isolations as well as desired DNA concentrations. An additional limitation was the limited time points that were observed in the experiment. Lastly, it should be noted that telomerase expression was not quantified in this experiment. There would be potential for increased telomerase levels to offset any telomere length damage that could take place during these time points.

While acknowledging limitations, the results of this work illuminate telomere dynamics between male and female *A. sagrei* as well as the effects of IGF manipulation in early life. These findings provide the groundwork for more extensive experiments to continue testing for potential effects of IGFs and sex on telomere dynamics across life. To expand upon this work, if the experiment were to be carried out up to and past sexual maturity, we know that telomere differences would be seen based on preliminary data. This would hypothetically allow for the illumination of at what time point sexual dimorphism would impact telomere lengths. This proposed experiment extension would take data points at 26, 39, and 52 weeks with the same injection treatments used here and would potentially allow for further insight into the impacts IGF-I and IGF-II may have in adulthood of *A. sagrei*. The addition of telomerase expression quantification would also allow for a better understanding of the rate of telomere attrition as well as telomere recovery rate.

Data Accessibility

Experimental data has not yet been published but can be found at:

TS_Lab_ AnoleAging > NIH_R15_Anole_ModelAging_IGFs > Aim1_LabLongevity_Aim > Telomeres_EarlyLife_Ryan > Telomere_Analysis_Code

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Code

Molecular Sexing and Telomere qPCR Analysis Pipeline

Ryan Hardin

December 14, 2022

Modified from “Gene expression qPCR Analysis Pipeline” by Tori Coutts

This is an R Markdown Notebook. When you execute code within the notebook, the results appear beneath the code.

Try executing this chunk by clicking the *Run* button within the chunk or by placing your cursor inside it and pressing *Ctrl+Shift+Enter*.

```
plot(cars)
```

Add a new chunk by clicking the *Insert Chunk* button on the toolbar or by pressing *Ctrl+Alt+I*.

When you save the notebook, an HTML file containing the code and output will be saved alongside it (click the *Preview* button or press *Ctrl+Shift+K* to preview the HTML file).

The preview shows you a rendered HTML copy of the contents of the editor. Consequently, unlike *Knit*, *Preview* does not run any R code chunks. Instead, the output of the chunk when it was last run in the editor is displayed.

###Load necessary packages

```
library(tidyverse)
```

```
Registered S3 methods overwritten by 'dbplyr':
```

```
  method         from
```

```
  print.tbl_lazy
```

```
  print.tbl_sql
```

```
── Attaching packages ─────────────────────────────────────── tidyverse 1.3.2 ──
✓ ggplot2 3.4.0      ✓ purrr   1.0.1
✓ tibble  3.1.8      ✓ dplyr   1.0.10
✓ tidyr   1.3.0      ✓ stringr 1.5.0
✓ readr   2.1.3      ✓ forcats 0.5.2 ── Conflicts ────────────────────────── tidyverse_conflicts() ──
✖ dplyr::filter() masks stats::filter()
✖ dplyr::lag()    masks stats::lag()
```

```
library(nlme)
```

```
Attaching package: 'nlme'

The following object is masked from 'package:dplyr':

    collapse
library(lme4)
Loading required package: Matrix

Attaching package: 'Matrix'

The following objects are masked from 'package:tidyr':

    expand, pack, unpack


Attaching package: 'lme4'

The following object is masked from 'package:nlme':

    lmList
library(ggplot2)
#library(HTqPCR)
#from Abby's code
library(ggplot2)
library(nlme)
library(multcomp)
Loading required package: mvtnorm
Loading required package: survival
Loading required package: TH.data
Loading required package: MASS

Attaching package: 'MASS'

The following object is masked from 'package:dplyr':

    select
```

```
Attaching package: 'TH.data'

The following object is masked from 'package:MASS':

    geyser
library(emmeans)
library(MuMIn)
library(bestNormalize)

Attaching package: 'bestNormalize'

The following object is masked from 'package:MASS':

    boxcox
library(lmtest)
Loading required package: zoo

Attaching package: 'zoo'

The following objects are masked from 'package:base':

    as.Date, as.Date.numeric
library(devtools)
Loading required package: usethis
Registered S3 method overwritten by 'htmlwidgets':
  method           from
  print.htmlwidget tools:rstudio

Attaching package: 'devtools'

The following object is masked from 'package:emmeans':

    test
library(ggpubr)
```

###Upload raw data and metadata files for Experiment 1

###Check for successful data upload for Experiment 1

```
head(All_Plate_Data)


summary(All_Plate_Data)
  Plate.Number       Well               ID             Treatment             Group
Cq_A
 Min.   :1.000   Length:177         Length:177         Length:177         Min.   :1
Min.   :20.97
 1st Qu.:1.000   Class :character   Class :character   Class :character   1st Qu.:1
1st Qu.:21.82
 Median :2.000   Mode  :character   Mode  :character   Mode  :character   Median :2
Median :22.13
 Mean   :1.898                                                           Mean   :2
Mean   :22.25
 3rd Qu.:3.000                                                           3rd Qu.:3
3rd Qu.:22.43
 Max.   :3.000                                                           Max.   :3
Max.   :27.34
      Sq_A              Cq_X            Sq_X              Cq_T            Sq_T
Sq_X_A
 Min.   :  82.84   Min.   :20.76   Min.   :  36.08   Min.   :15.48   Min.   :  1295
Min.   :0.4091
 1st Qu.:1511.10   1st Qu.:21.69   1st Qu.: 848.54   1st Qu.:16.59   1st Qu.: 49957
1st Qu.:0.4875
 Median :1857.40   Median :22.18   Median :1330.48   Median :16.91   Median : 67173
Median :0.8091
 Mean   :1906.84   Mean   :22.38   Mean   :1370.63   Mean   :17.06   Mean   : 69850
Mean   :0.7185
 3rd Qu.:2270.22   3rd Qu.:23.01   3rd Qu.:1886.78   3rd Qu.:17.35   3rd Qu.: 87623
3rd Qu.:0.8736
 Max.   :4145.87   Max.   :28.21   Max.   :2990.73   Max.   :22.33   Max.   :173085
Max.   :1.2057
     Cq_X_A       Sq_Average_X_A   Cq_Average_X_A       Sex                Sq_T_A
 Min.   :0.9597   Min.   :0.4337   Min.   :0.9645   Length:177         Min.   :  1.022
 1st Qu.:0.9867   1st Qu.:0.4828   1st Qu.:0.9872   Class :character   1st Qu.: 31.514
 Median :0.9928   Median :0.8099   Median :0.9920   Mode  :character   Median : 34.349
 Mean   :1.0056   Mean   :0.7185   Mean   :1.0056                      Mean   : 37.226
 3rd Qu.:1.0322   3rd Qu.:0.8751   3rd Qu.:1.0329                      3rd Qu.: 39.089
 Max.   :1.0435   Max.   :1.1343   Max.   :1.0412                      Max.   :145.988
     Cq_T_A       Sq_Average_T_A   Cq_Average_T_A
 Min.   :0.6898   Min.   : 11.27   Min.   :0.6917
 1st Qu.:0.7583   1st Qu.: 31.43   1st Qu.:0.7590
```

```
 Median :0.7668   Median : 34.53   Median :0.7660
 Mean   :0.7665   Mean   : 37.23   Mean   :0.7665
 3rd Qu.:0.7766   3rd Qu.: 38.44   3rd Qu.:0.7755
 Max.   :0.9809   Max.   :141.75   Max.   :0.8698
```

#Some QC was performed in Excel before files were imported All raw qPCR data files were first imported to excel and mergec into one main file. Sex was then calculated using a formula (IFS(##<0.66, “M”, ##>0.82, “F”) using the sq (starting quantity) ratio of x-linked/autosmal gene ratio in Excel.

These data are refered to Experiment 1 relative to the chapter in the MS thesis by Ryan Hardin. Final_Altered_Threshold_All_Plates_qPCR_Information_12_12_22

###If merging data is not performed in Excel, use code below for Experiment 1

```
##MergedData=merge(rawdata,metadata,by="Well")
##Hardin data merged in Excel
```

###Ensure the merge was successful

```
##head(mergedfile)
```

#Change Sex and Treatment to factors

#Test Experiment one to determine if the copy number of the reference gene significantly different between treatment groups

```
orq=orderNorm(All_Plate_Data$Sq_A)
Final_Plate_Data=cbind(All_Plate_Data, orq=orq$x.t)
model.Adarb2=lme(orq~Treatment, random =  ~1|Plate.Number/ID, data=Final_Plate_Data,na
.action=na.exclude)
summary(model.Adarb2)
Linear mixed-effects model fit by REML
  Data: Final_Plate_Data

Random effects:
 Formula: ~1 | Plate.Number
        (Intercept)
StdDev:   0.4650944

 Formula: ~1 | ID %in% Plate.Number
        (Intercept)  Residual
StdDev:   0.8989448 0.2252141
```

```
Fixed effects:  orq ~ Treatment
 Correlation:
                (Intr) TrtmD7
TreatmentDay7   -0.419
TreatmentEmbryo -0.397  0.477

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-4.12082813 -0.32709071  0.01224614  0.33315382  4.54962711

Number of Observations: 183
Number of Groups:
        Plate.Number ID %in% Plate.Number
                   3                   61
anova(model.Adarb2)
#autosomal gene sig difference between treatment test below
lsmeans(model.Adarb2, pairwise ~ Treatment)
$lsmeans
 Treatment  lsmean    SE df lower.CL upper.CL
 Day1      -0.1340 0.333  2    -1.57     1.30
 Day7      -0.0796 0.338  2    -1.53     1.38
 Embryo     0.2875 0.345  2    -1.20     1.77

Degrees-of-freedom method: containment
Confidence level used: 0.95

$contrasts
 contrast      estimate    SE df t.ratio p.value
 Day1 - Day7    -0.0545 0.290 56  -0.188  0.9808
 Day1 - Embryo  -0.4216 0.291 56  -1.448  0.3236
 Day7 - Embryo  -0.3671 0.297 56  -1.234  0.4381

Degrees-of-freedom method: containment
P value adjustment: tukey method for comparing a family of 3 estimates
orq_T=orderNorm(All_Plate_Data$Sq_T_A)
Final_Plate_Data=cbind(All_Plate_Data, orq_T=orq_T$x.t)
```

```
model.Telomere=lme(orq_T~Treatment * Sex, random =  ~1|Plate.Number/ID, data=Final_Pla
te_Data,na.action=na.exclude)
summary(model.Telomere)
Linear mixed-effects model fit by REML
  Data: Final_Plate_Data

Random effects:
 Formula: ~1 | Plate.Number
        (Intercept)
StdDev:  0.05651818

 Formula: ~1 | ID %in% Plate.Number
        (Intercept)  Residual
StdDev:    0.970077 0.3499204

Fixed effects:  orq_T ~ Treatment * Sex
 Correlation:
                     (Intr) TrtmD7 TrtmnE SexM   TD7:SM
TreatmentDay7        -0.729
TreatmentEmbryo      -0.651  0.481
SexM                 -0.685  0.506  0.451
TreatmentDay7:SexM    0.405 -0.556 -0.266 -0.593
TreatmentEmbryo:SexM  0.456 -0.337 -0.700 -0.662  0.392

Standardized Within-Group Residuals:
          Min            Q1           Med            Q3           Max
-2.5778473631 -0.4937129599 -0.0009175944  0.4687564079  3.8761053406

Number of Observations: 177
Number of Groups:
        Plate.Number ID %in% Plate.Number
                   3                   59
anova.lme(model.Telomere)
#Experiment one test for differences in telomere length between sex and treatment
orq_T=orderNorm(All_Plate_Data$Sq_T_A)
Final_Plate_Data=cbind(All_Plate_Data, orq_T=orq_T$x.t)
```

```
model.Telomere=lme(orq_T~Treatment + Sex, random =  ~1|Plate.Number/ID, data=Final_Pla
te_Data,na.action=na.exclude)

summary(model.Telomere)

anova.lme(model.Telomere)

lsmeans(model.Telomere, pairwise ~ Sex)

lsmeans(model.Telomere, pairwise ~ Treatment)
```

###Experiment 1 graph code ###Plotting sex for Experiment 1

```
#Calculate average copy number for each age and gene per individual. This averages acr
oss qpcr triplicates and provides SD

###Sexing

Sexing.SQ=summary(Final_Plate_Data, measurevar = "Sq_X_A", groupvars = c("Sex"))

Final.Sexing.SQ <- Sexing.SQ [-1,]

###Telomere

Telomere.SQ=summary(Final_Plate_Data, measurevar = "Sq_T", groupvars =c("Treatment", "
Sex"))

Final.Telomere.SQ <- Telomere.SQ [-3,]

Final_Plate_Data2 <- Final_Plate_Data [-c(13,14,15,31,32,33),]

#plot copy number by age. This plot is used as an inset in the plot including all time
points. These are difficult to see in the embryonic-adult graph as the adult expressio
n is significantly higher than any other timepoint.
```

###Experiment 1 graph code ###Plotting sex for Experiment 1

```
### Plot sex for experiment 1

Final_Plate_Data2$Treatment<- as.factor(Final_Plate_Data2$Sq_X_A)


p3=ggplot(Final_Plate_Data2, aes(x=Sex, y=Sq_X_A, fill= Sex)) +

  ylab("Relative Copy Number") +

 xlab("Sex") +

 ggtitle("Sex Determination") +

 geom_violin(trim=F, position= "dodge", scale="area") +

  scale_fill_manual(values = c("orange2", "skyblue4")) +

 geom_boxplot(width=0.2, position= "dodge", outlier.shape = NA, color="black") +

#geom_point(data = Final.Sexing.SQ, size =2, shape = 19, color="black", position=posit
ion_dodge(width=0.9)) +

geom_point(position=position_jitterdodge(jitter.width = 0.03, dodge.width = 0.9), size
=1, alpha=0.5, aes(group= Sex), color="black") +

  #scale_y_continuous(labels="fancy_scientific") +

 theme(axis.text.x = element_text(size=12))
```

```
p3

#Save file as PNG for final figure production in BioRender

#

ggsave(p3, file="Experiment1A.nc.png", width=9, height=7, dpi=600)
```

###Plotting Telomere/Sex analysis for Experiment 1

```
###Telomere/Sex analysis for Experiment 1

Final_Plate_Data2$Treatment<- factor(Final_Plate_Data2$Treatment, levels = c("Embryo",
"Day1", "Day7"))


p2=ggplot(Final_Plate_Data2, aes(x=Treatment, y=Sq_T, fill= Sex)) +

  ylab("Relative Copy Number") +

 xlab("Life Stage") +

 ggtitle("Telomeres Across Early Life") +

 geom_violin(trim=F, position= "dodge", scale="area") +

  scale_fill_manual(values = c("orange2", "skyblue4")) +

 geom_boxplot(width=0.2, position= "dodge", outlier.shape = NA, color="black") +

#geom_point(data = Final.Telomere.SQ, size =2, shape = 19, color="black", position=pos
ition_dodge(width=0.9)) +

geom_point(position=position_jitterdodge(jitter.width = 0.05, dodge.width = 0.9), size
=1, alpha=0.5, aes(group= Sex), color="black") +

  #scale_y_continuous(labels="fancy_scientific") +

 theme(axis.text.x = element_text(size=12))

p2

#Save file as PNG for final figure production in BioRender

#

ggsave(p2, file="Experiment1B.nc.png", width=9, height=7, dpi=600)
```

~~~~~~~~~~

```
#Calculate average copy number for each age and gene per individual. This averages acr
oss qpcr triplicates and provides SD

###Sexing

Sexing.SQ=summarySE(All_Plate_Data, measurevar = "Sq_X_A", groupvars = c("Sex"))

Final.Sexing.SQ <- Sexing.SQ [-1,]

###Telomere

Telomere.SQ=summarySE(Final_Plate_Data, measurevar = "Sq_T", groupvars =c("Treatment",
"Sex"))

Final.Telomere.SQ <- Telomere.SQ [-3,]

Final_Plate_Data2 <- Final_Plate_Data [-c(13,14,15,31,32,33),]
```
~~~~~~~~~~

```
#plot copy number by age. This plot is used as an inset in the plot including all time
points. These are difficult to see in the embryonic-adult graph as the adult expressio
n is significantly higher than any other timepoint.
```

Code

Telomere Analysis for Aging anoles Week 1 to Week 13

Ryan Hardin

July 2023

###Load necessary packages

```
library(tidyverse)
Registered S3 methods overwritten by 'dbplyr':
  method         from
  print.tbl_lazy
  print.tbl_sql
── Attaching packages ─────────────────────────────────────── tidyverse
1.3.2 ──✓ ggplot2 3.4.0      ✓ purrr   1.0.1
✓ tibble  3.1.8      ✓ dplyr   1.0.10
✓ tidyr   1.3.0      ✓ stringr 1.5.0
✓ readr   2.1.3      ✓ forcats 0.5.2 ── Conflicts ──────────────────────────
──────────────────── tidyverse_conflicts() ──
✖ dplyr::filter() masks stats::filter()
✖ dplyr::lag()    masks stats::lag()
library(nlme)

Attaching package: 'nlme'

The following object is masked from 'package:dplyr':

    collapse
library(lme4)
Loading required package: Matrix

Attaching package: 'Matrix'

The following objects are masked from 'package:tidyr':
```

```
    expand, pack, unpack


Attaching package: 'lme4'

The following object is masked from 'package:nlme':

    lmList
library(ggplot2)
#library(HTqPCR)
#from Abby's code
library(ggplot2)
library(nlme)
library(multcomp)
Loading required package: mvtnorm
Loading required package: survival
Loading required package: TH.data
Loading required package: MASS

Attaching package: 'MASS'

The following object is masked from 'package:dplyr':

    select


Attaching package: 'TH.data'

The following object is masked from 'package:MASS':

    geyser
library(emmeans)
library(MuMIn)
library(bestNormalize)

Attaching package: 'bestNormalize'
```

```
The following object is masked from 'package:MASS':

    boxcox
library(lmtest)
Loading required package: zoo

Attaching package: 'zoo'

The following objects are masked from 'package:base':

    as.Date, as.Date.numeric
library(devtools)
Loading required package: usethis
Registered S3 method overwritten by 'htmlwidgets':
  method           from
  print.htmlwidget tools:rstudio

Attaching package: 'devtools'

The following object is masked from 'package:emmeans':

    test
library(ggpubr)
#library(NormqPCR)
```

Upload Copy Number Data and metadata files.

The datafile was output from the BioRad qPCR software program (Maestro).

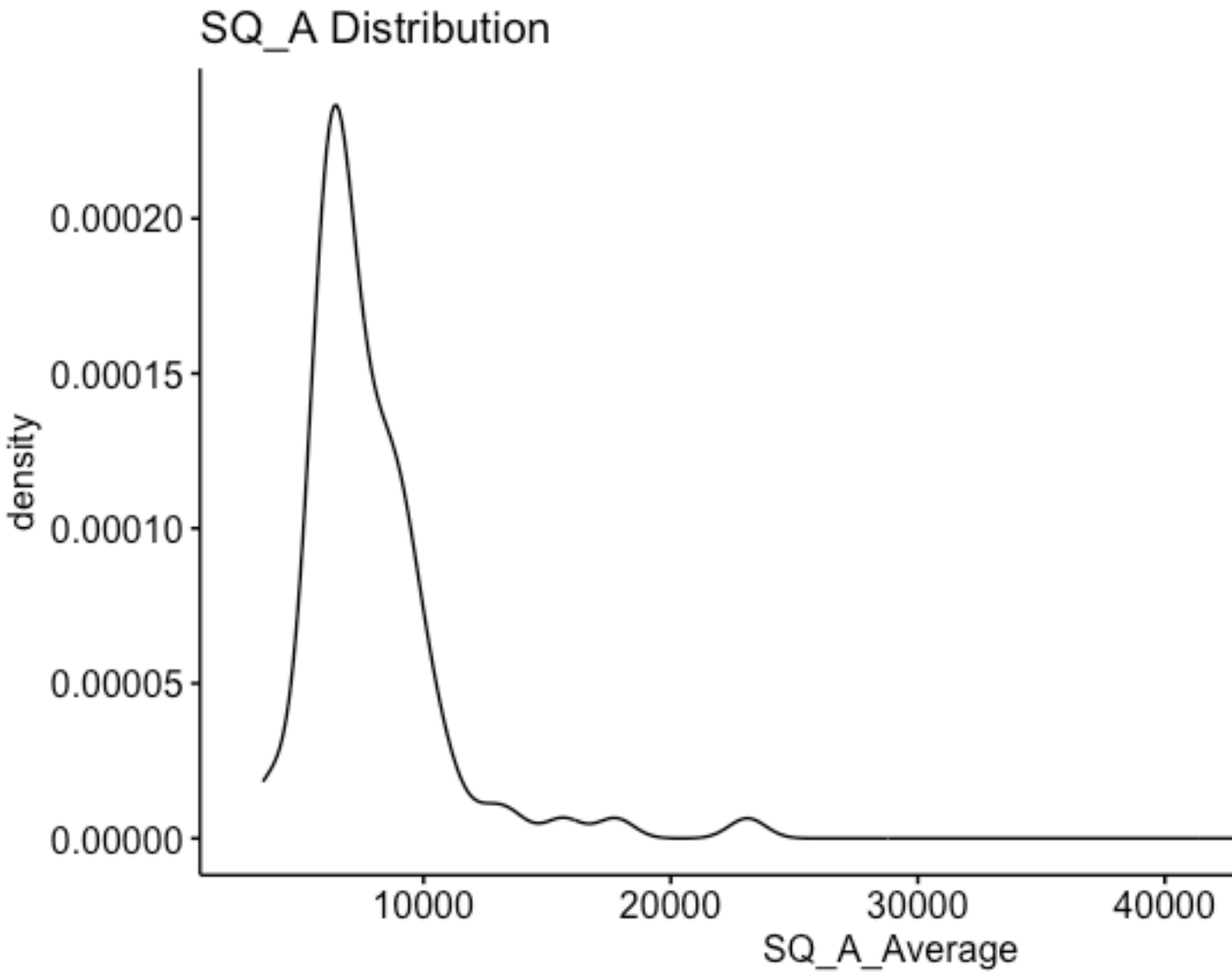
QC was largely performed in Excel before files were imported All raw qPCR data files were first imported to excel. With all samples being ran in triplicate, it was important to identify and remove any outliers of the x-linked and autosomal genes as well as the telomeres. To do this I averaged the cq value (quantification cycle) for the triplicates of a sample and then looked at the distance an individual triplicate would be away from the triplicate average. If the value was greater than 0.3 for x-linked or autosomal, it would be removed from the triplicates and averaged again. I also calculated standard error, standard deviation, and confidence limits for both x-linked and autosomal genes. Sex was then calculated using a formula (IFS(##<0.66, “M”, ##>0.82, “F”) using the sq (starting quantity) ratio of x-linked/autosmal gene ratio. Quality control for telomeres was conducted in the same way with the exception of the value for being removed from triplicates was increased from 0.3 to 0.4 due to the less consistent nature of the telomere qPCR reaction.

These data are refered to Experiment 2 relative to the chapter in the MS thesis by Ryan Hardin. Experiment_2_Working_File_F. Experiment_2_Sexing_Sq.

```
##Experiment 2 data file upload
Experiment_2_Data=read.csv(file.choose())
Error in file.choose() : file choice cancelled
```

Check for successful data upload for Experiment 2

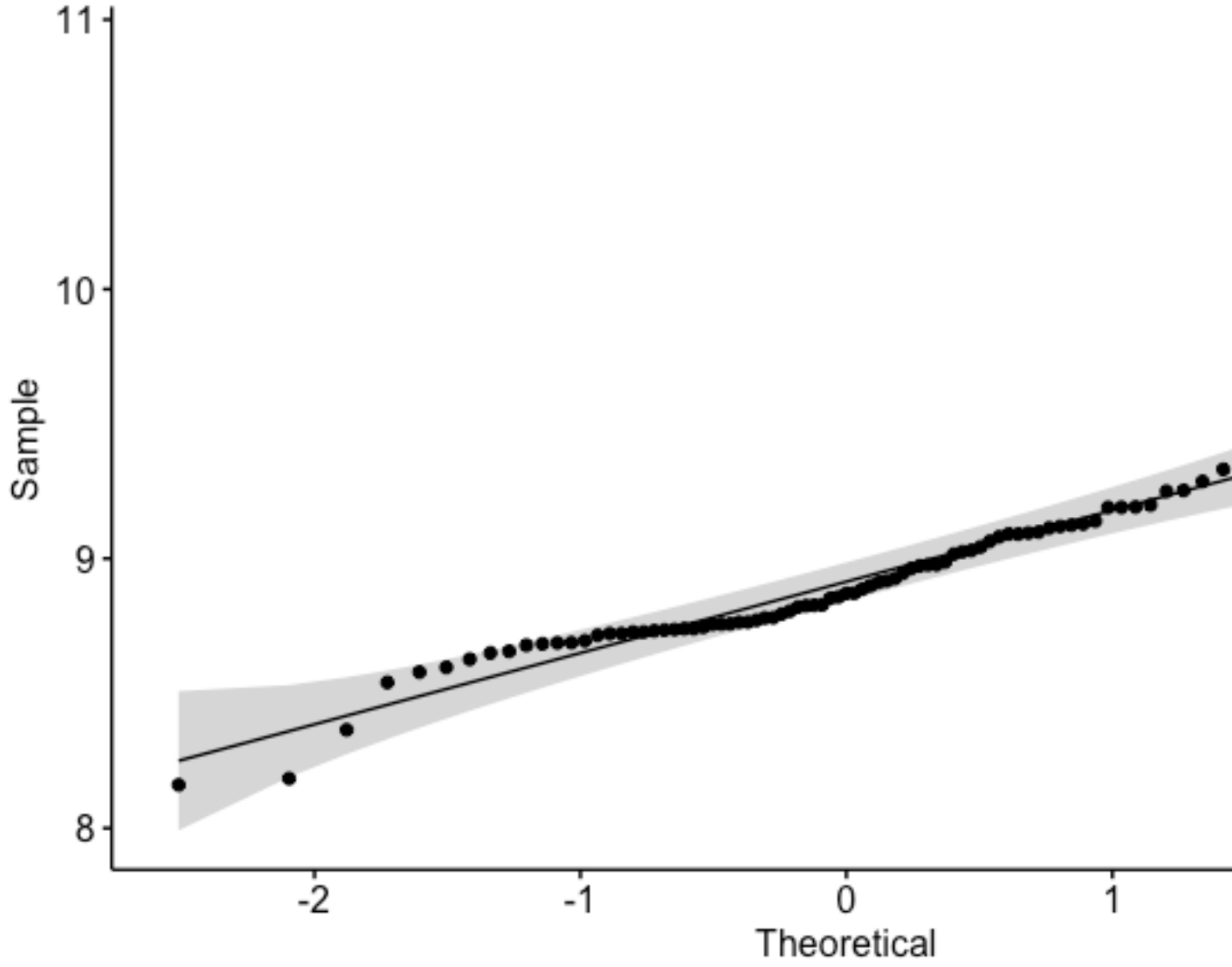
Test autosomal gene for normality




```
ggdensity(Experiment_2_Data$Sq_A_SampleAvg,
```

```
          main = "SQ_A Distribution",
          xlab = "SQ_A_Average")


mean(Experiment_2_Data$Sq_A_SampleAvg)
[1] 8406.368
sd(Experiment_2_Data$Sq_A_SampleAvg)
[1] 5851.148
```

Normalize Autosomal with Log Transformation



###Test if autosomal gene differs between treatments

```
##Experiment two testing for differences in control gene, ADARB2


#Differences between treatments
model.Adarb2_Treatment=lme(Log_A_Average ~ Treatment, random = ~1|Plate_Number, data=E
xperiment_2_Data, na.action = na.exclude)
summary(model.Adarb2_Treatment)
Linear mixed-effects model fit by REML
  Data: Experiment_2_Data

Random effects:
 Formula: ~1 | Plate_Number
        (Intercept)  Residual
StdDev:  7.7781e-06 0.3698317

Fixed effects:  Log_A_Average ~ Treatment
 Correlation:
                (Intr) TrtmnO TrtmnP
TreatmentOrange -0.699
TreatmentPink   -0.681  0.476
TreatmentYellow -0.699  0.488  0.476

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-2.0136479 -0.5530874 -0.2109821  0.1946609  5.0023887

Number of Observations: 83
Number of Groups: 4
model.Adarb2_Treatment_anova = anova(model.Adarb2_Treatment)
summary(model.Adarb2_Treatment_anova)
     numDF         denDF       F-value           p-value
 Min.   :1.0   Min.   :76   Min.   :    1.2   Min.   :0.0000
 1st Qu.:1.5   1st Qu.:76   1st Qu.:12131.2   1st Qu.:0.0791
 Median :2.0   Median :76   Median :24261.3   Median :0.1582
 Mean   :2.0   Mean   :76   Mean   :24261.3   Mean   :0.1582
 3rd Qu.:2.5   3rd Qu.:76   3rd Qu.:36391.3   3rd Qu.:0.2373
 Max.   :3.0   Max.   :76   Max.   :48521.3   Max.   :0.3164
sink("Adarb2_lme_Treatment_anova.txt")
```

```
  summary(model.Adarb2_Treatment)
  summary(model.Adarb2_Treatment_anova)
on.exit(close(sink))
```

###Test if autosomal gene differs between sexes

```
#Differences between sexes
model.Adarb2_Sex=lme(Log_A_Average ~ Sex_Sq, random = ~1|Plate_Number, data=Experiment_2_Data, na.action = na.exclude)
summary(model.Adarb2_Sex)
model.Adarb2_Sex_anova = anova(model.Adarb2_Sex)
summary(model.Adarb2_Sex_anova)
sink("Adarb2_lme_Sex_anova.txt")
  summary(model.Adarb2_Sex)
  summary(model.Adarb2_Sex_anova)
on.exit(close(sink))
```

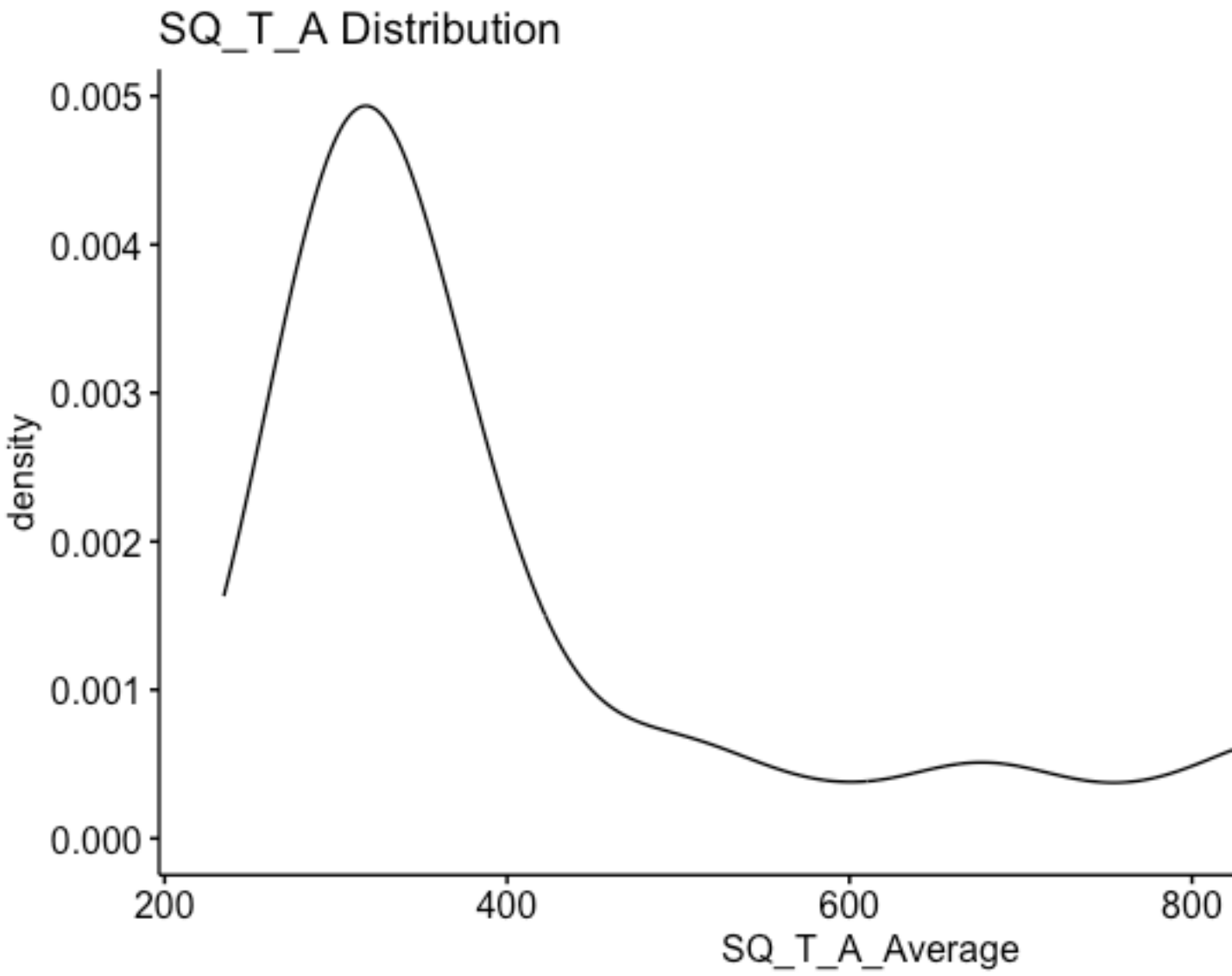
###Test if autosomal gene differs between age

```
#Differences between age
model.Adarb2_Age=lme(Log_A_Average ~ Age, random = ~1|Plate_Number, data=Experiment_2_Data, na.action = na.exclude)
summary(model.Adarb2_Age)
model.Adarb2_Age_anova = anova(model.Adarb2_Age)
summary(model.Adarb2_Age_anova)
sink("Adarb2_lme_Age_anova.txt")
  summary(model.Adarb2_Age)
  summary(model.Adarb2_Age_anova)
on.exit(close(sink))
```

Telomere/Autosomal Analysos

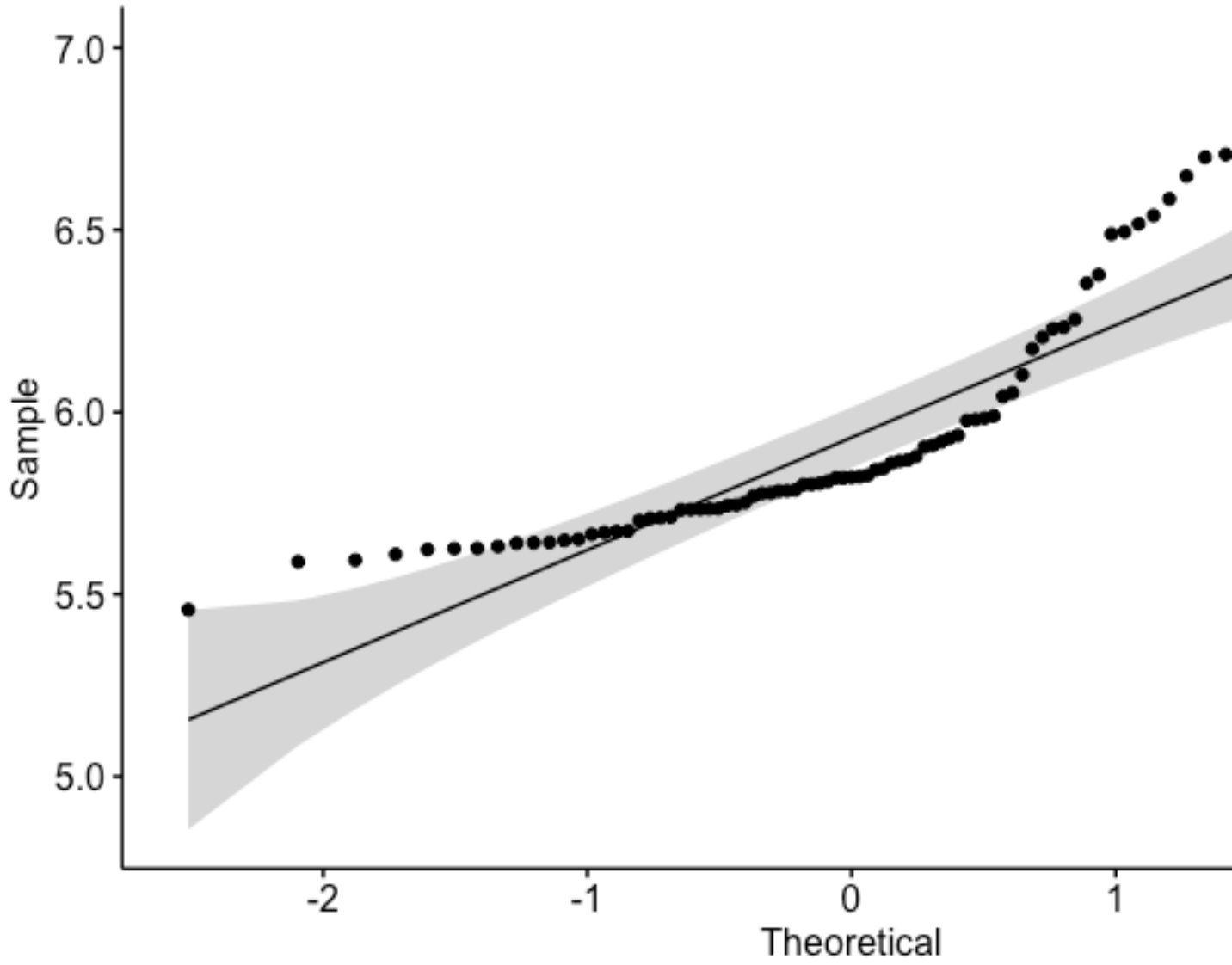
Test if Telomere/Autosmal value is normally Distributed: SQ_T_A_Average data

```
ggdensity(Experiment_2_Data$Sq_T_A_Average,
          main = "SQ_T_A Distribution",
          xlab = "SQ_T_A_Average")
```




```
mean(Experiment_2_Data$Sq_T_A_Average)
sd(Experiment_2_Data$Sq_T_A_Average)
```

Normalize Telomere/Autosomal with Log Transformation



Plot Sexes

```
p2=ggplot(Experiment_2_Sexing_Data, aes(x=Sex_Sq, y=Sq_Average_X_A, fill= Sex_Sq)) +
  ylab("X-linked to autosomal gene copy ratio") +
 xlab("Sex") +
 ggtitle("Sex Validation") +
 geom_violin(trim=F, position= "dodge", scale="width", adjust = 0.60) +
  scale_fill_manual(values = c("orange2", "skyblue4")) +
  geom_boxplot(width=0.3, position= "dodge", outlier.shape = NA, color="black") +
 #geom_point(data = Sq_T_SampleAvg, size =2, shape = 19, color="black", position=posit
ion_dodge(width=0.9)) +
```

```
 geom_point(position=position_jitterdodge(jitter.width = 0.05, dodge.width = 0.9), siz
e=1, alpha=0.5, aes(group= Sex_Sq), color="black") +

  #scale_y_continuous(labels="fancy_scientific") +

 theme(axis.text.x = element_text(size=12))

p2

#Save file as PNG for final figure production in BioRender

#

ggsave(p2, file="Sample_Sex_Validation.png", width=9, height=7, dpi=600)
```

Question 1. Is there an effects of the IGF injection treatments at 13 weeks on Telomere length in either sex?

Use the data from Week 13 only since these animals received an injection (week 1 did not. Treatment groups are: Orange, Yellow, Pink Linear tests using log transformed Sq_T_A_Average

Subset week 13 data below

```
#Differences in telomere length between sexes and injection treatments at 13 weeks

Experiment_2_Data_Week_7 <- subset(Experiment_2_Data, Age == "Week_7")
```

Testing for the interaction of IGF injection treatments and sex, and their interaction on telomere length at week 13

```
model.TSInt_lme = lme(Log_Sq_Average ~ Treatment * Sex_Sq * Mass_Change_1_13, random =
~1|Plate_Number, data=Experiment_2_Data_Week_13, na.action = na.exclude)

summary(model.TSInt_lme)


anova(model.TSInt_lme)
```

Question 2. Does sex and IGF injection affect telomere length at 13 Weeks?

Testing for individual effects of treatment and Sex_Sq as independent variables, using a lnear mixed effects model on Week 13

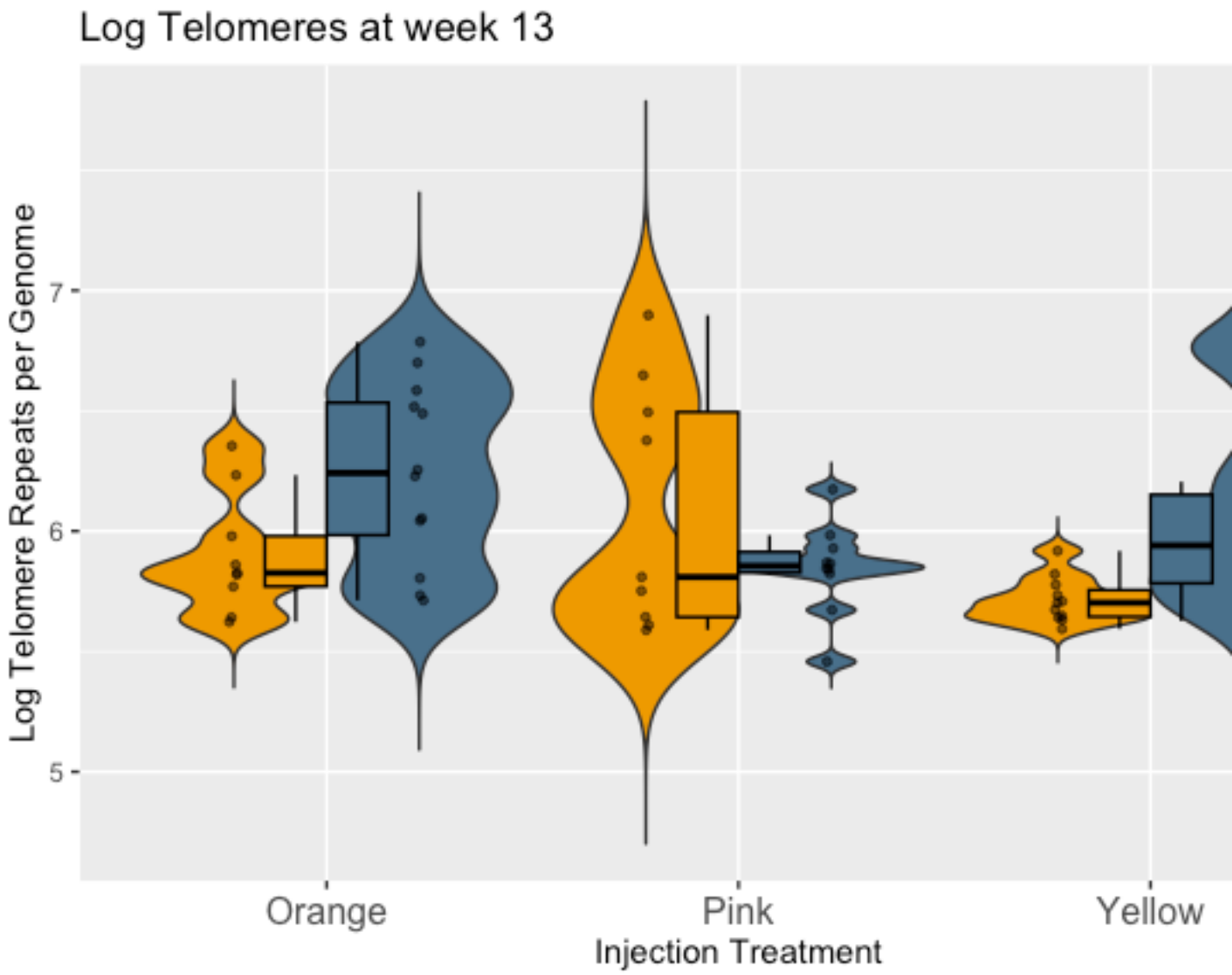
```
model.TSInd_lme = lme(Log_Sq_Average ~ Treatment + Sex_Sq, random = ~1|Plate_Number, d
ata=Experiment_2_Data_Week_13, na.action = na.exclude)
```

```
summary(model.TSInd_lme)


anova(model.TSInd_lme)
```

###lsmeans calculations for sex, treatment, and treatment by sex for both lm and lme models

```
summary(model.TSInd_lme)
Ind.Sex = emmeans(model.TSInd_lme, pairwise ~ Sex_Sq)
Ind.Treatment = emmeans(model.TSInd_lme, pairwise ~ Treatment)
Ind.Treatment.Sex = emmeans(model.TSInd_lme, pairwise ~ Treatment + Sex_Sq)

###The following does not work due to results being misleading due to interaction
Int.Sex = emmeans(model.TSInt_lme, pairwise ~ Sex_Sq)
Int.Treatment = emmeans(model.TSInt_lme, pairwise ~ Treatment)
Int.Treatment.Sex = emmeans(model.TSInt_lme, pairwise ~ Treatment * Sex_Sq)
```

###Plotting Week 13 analysis looking at LOG telomere lengths across injection treatments and between sexes



~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Question 3. Is there an effect of Growth, Treatment, or Sex on Telomere length?

LME of Treatment by Sex by Mass_Change on Log Average of Telomere length
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

Model using sex to explain whether or not sex has a significant effect on /

telomere length

```
lmeWithSex = lme(Log_Sq_Average ~ relevel(Treatment, r = "Pink") * Mass_Change_1_13 *
Sex_Sq , random = ~1|Plate_Number, data=Experiment_2_Data_Week_13, na.action = na.excl
ude, method = "REML")

summary(lmeWithSex)

Linear mixed-effects model fit by REML

  Data: Experiment_2_Data_Week_13


Random effects:

 Formula: ~1 | Plate_Number

        (Intercept)  Residual

StdDev:   0.3260261 0.1599358


Fixed effects:  Log_Sq_Average ~ relevel(Treatment, r = "Pink") * Mass_Change_1_13 *
Sex_Sq

 Correlation:

                                                                        (Intr) rl(T,r="P")O rl(T
,r="P")Y Ms_C_1_13 Sx_SqM

relevel(Treatment, r = "Pink")Orange                                    -0.441

relevel(Treatment, r = "Pink")Yellow                                    -0.495  0.375

Mass_Change_1_13                                                        -0.730  0.543        0.6
24

Sex_SqM                                                                 -0.533  0.404        0.4
62        0.671

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13                    0.419 -0.970       -0.3
63       -0.565    -0.385

relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13                    0.466 -0.338       -0.9
66       -0.643    -0.436

relevel(Treatment, r = "Pink")Orange:Sex_SqM                             0.324 -0.746       -0.2
89       -0.394    -0.609

relevel(Treatment, r = "Pink")Yellow:Sex_SqM                             0.304 -0.259       -0.6
41       -0.367    -0.599

Mass_Change_1_13:Sex_SqM                                                 0.460 -0.330       -0.4
12       -0.635    -0.953

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13:Sex_SqM -0.300  0.686        0.2
80        0.405     0.617
```

```
relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13:Sex_SqM -0.270  0.219        0.5
95        0.362     0.594

                                                                    rl(T,r="P")O:M_C_1_13 rl
(T,r="P")Y:M_C_1_13 r(T,r="P")O:S

relevel(Treatment, r = "Pink")Orange

relevel(Treatment, r = "Pink")Yellow

Mass_Change_1_13

Sex_SqM

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13

relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13          0.361

relevel(Treatment, r = "Pink")Orange:Sex_SqM                   0.722                 0
.253

relevel(Treatment, r = "Pink")Yellow:Sex_SqM                   0.236                 0
.602                 0.402

Mass_Change_1_13:Sex_SqM                                       0.351                 0
.427                 0.561

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13:Sex_SqM -0.710                -0
.275                -0.963

relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13:Sex_SqM -0.221                -0
.602                -0.381

                                                                    r(T,r="P")Y:S M_C_1_13:
r(T,r="P")O:M_C_1_13:

relevel(Treatment, r = "Pink")Orange

relevel(Treatment, r = "Pink")Yellow

Mass_Change_1_13

Sex_SqM

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13

relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13

relevel(Treatment, r = "Pink")Orange:Sex_SqM

relevel(Treatment, r = "Pink")Yellow:Sex_SqM

Mass_Change_1_13:Sex_SqM                                        0.557

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13:Sex_SqM -0.388        -0.633

relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13:Sex_SqM -0.968        -0.612
0.409


Standardized Within-Group Residuals:

        Min          Q1         Med          Q3         Max

-2.15278682 -0.59706097 -0.02229026  0.48108923  2.18286095


Number of Observations: 61
```

```
Number of Groups: 4
```

#Look for confidence interval violations

```
intervals(lmeWithSex)
```

#No upper-lower pairs crossed 0, i.e. No positive and negative paired values #No violations of CIs, model should still be good

Model without sex to explain if treatment and mass has a significant effect /

on telemere length

```
lmeWithoutSex = lme(Log_Sq_Average ~ relevel(Treatment, r = "Pink") + Mass_Change_1_13
+ Sex_Sq + Mass_Change_1_13:relevel(Treatment, r = "Pink"), random = ~1|Plate_Number,
data=Experiment_2_Data_Week_13, na.action = na.exclude, method = "REML")

summary(lmeWithoutSex)

Linear mixed-effects model fit by REML

  Data: Experiment_2_Data_Week_13


Random effects:

 Formula: ~1 | Plate_Number

        (Intercept) Residual

StdDev:   0.3457203 0.161266


Fixed effects:  Log_Sq_Average ~ relevel(Treatment, r = "Pink") + Mass_Change_1_13 +
Sex_Sq + Mass_Change_1_13:relevel(Treatment, r = "Pink")

 Correlation:

                                                    (Intr) rl(T,r="P")O rl(T,r="P")Y
M_C_1_ Sx_SqM r(T,r="P")O:

relevel(Treatment, r = "Pink")Orange                -0.376

relevel(Treatment, r = "Pink")Yellow                -0.384  0.377

Mass_Change_1_13                                    -0.598  0.588        0.594

Sex_SqM                                             -0.212  0.073        0.073
0.197

relevel(Treatment, r = "Pink")Orange:Mass_Change_1_13  0.369 -0.970       -0.366
-0.624 -0.089

relevel(Treatment, r = "Pink")Yellow:Mass_Change_1_13  0.379 -0.373       -0.972
-0.632 -0.080  0.391
```

```
Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max 
-2.30074479 -0.58403117 -0.05966436  0.53230063  2.34675158 

Number of Observations: 61
Number of Groups: 4
```

#Look for confidence interval violations

```
intervals(lmeWithSex)
```

#No upper-lower pairs crossed 0, i.e. No positive and negative paired values #No violations of CIs, model should still be good

Figure 2: Telomere ~ Rx:Mass

```
Fig2 <- ggplot(Experiment_2_Data_Week_13, aes(x = Mass_Change_1_13, y = Log_Sq_Average
, color = interaction(Sex_Sq, Treatment))) +
  ylab("Log Telomere Length") +
  xlab("Mass change (g)") +
  ggtitle("Effect of Mass:Treatment on Telomeres") +
  #geom_point() +
  geom_smooth(method = "lm", se = FALSE) +
  scale_color_manual(values = c("orange1", "orange3", "pink1", "pink3", "yellow1", "ye
llow3"))
# Add more colors if you have more combinations of Sex and Treatment


Fig2
```

###Telomere/Sex analysis for Experiment 2 plotting only Week 13 data

```
Experiment_2_Data_Week_13$Treatment<- factor(Experiment_2_Data_Week_13$Treatment, leve
ls = c("Orange", "Pink", "Yellow"))


Sq_T_SampleAvg=summary(Experiment_2_Data_Week_13, measurevar = "Sq_T_A_Average", group
vars =c("Treatment", "Sex_Sq"))
```

Figure: Telomeres~SVL by Treatment by Sex

```
lmeWithSex = lme(Log_Sq_Average ~ relevel(Treatment, r = "Pink") * SVL_Change_1_13 * S
ex_Sq , random = ~1|Plate_Number, data=Experiment_2_Data_Week_13, na.action = na.exclu
de, method = "REML")

summary(lmeWithSex)
```

```
Linear mixed-effects model fit by REML
  Data: Experiment_2_Data_Week_13


Random effects:
 Formula: ~1 | Plate_Number
        (Intercept)  Residual
StdDev:   0.3510313 0.1673857


Fixed effects:  Log_Sq_Average ~ relevel(Treatment, r = "Pink") * SVL_Change_1_13 *
Sex_Sq
 Correlation:
                                                                    (Intr) rl(T,r="P")O rl(T,
r="P")Y SVL_Ch_1_13 Sx_SqM
relevel(Treatment, r = "Pink")Orange                                -0.531
relevel(Treatment, r = "Pink")Yellow                                -0.595  0.440
SVL_Change_1_13                                                     -0.828  0.609        0.68
8
Sex_SqM                                                             -0.622  0.464        0.51
9        0.719
relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13                 0.501 -0.983       -0.41
4       -0.599      -0.437
relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13                 0.573 -0.414       -0.97
8       -0.693      -0.498
relevel(Treatment, r = "Pink")Orange:Sex_SqM                         0.406 -0.749       -0.33
2       -0.466      -0.631
relevel(Treatment, r = "Pink")Yellow:Sex_SqM                         0.365 -0.306       -0.59
8       -0.412      -0.582
SVL_Change_1_13:Sex_SqM                                              0.554 -0.402       -0.46
2       -0.671      -0.971
relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13:Sex_SqM -0.366  0.697        0.29
9        0.441       0.616
relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13:Sex_SqM -0.335  0.275        0.55
8        0.395       0.584
                                                                    rl(T,r="P")O:SVL_C_1_13 r
l(T,r="P")Y:SVL_C_1_13
relevel(Treatment, r = "Pink")Orange
relevel(Treatment, r = "Pink")Yellow
SVL_Change_1_13
Sex_SqM
relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13
relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13          0.407
```

```
relevel(Treatment, r = "Pink")Orange:Sex_SqM                    0.738
0.313

relevel(Treatment, r = "Pink")Yellow:Sex_SqM                    0.280
0.571

SVL_Change_1_13:Sex_SqM                                        0.397
0.467

relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13:Sex_SqM -0.713                  -
0.297

relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13:Sex_SqM -0.262                  -
0.556

                                                                  r(T,r="P")O:S_ r(T,r="P")
Y:S_ SVL_C_1_13:

relevel(Treatment, r = "Pink")Orange

relevel(Treatment, r = "Pink")Yellow

SVL_Change_1_13

Sex_SqM

relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13

relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13

relevel(Treatment, r = "Pink")Orange:Sex_SqM

relevel(Treatment, r = "Pink")Yellow:Sex_SqM                    0.387

SVL_Change_1_13:Sex_SqM                                        0.605          0.548

relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13:Sex_SqM -0.979         -0.358
-0.628

relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13:Sex_SqM -0.382         -0.984
-0.584

                                                                  r(T,r="P")O:SVL_C_1_13:

relevel(Treatment, r = "Pink")Orange

relevel(Treatment, r = "Pink")Yellow

SVL_Change_1_13

Sex_SqM

relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13

relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13

relevel(Treatment, r = "Pink")Orange:Sex_SqM

relevel(Treatment, r = "Pink")Yellow:Sex_SqM

SVL_Change_1_13:Sex_SqM

relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13:Sex_SqM

relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13:Sex_SqM  0.375


Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
```

```
-2.23903512 -0.50814928  0.01955555  0.51987743  2.30559824


Number of Observations: 61

Number of Groups: 4

lmeWithoutSex = lme(Log_Sq_Average ~ relevel(Treatment, r = "Pink") + SVL_Change_1_13
+ Sex_Sq + SVL_Change_1_13:relevel(Treatment, r = "Pink"), random = ~1|Plate_Number, d
ata=Experiment_2_Data_Week_13, na.action = na.exclude, method = "REML")

summary(lmeWithoutSex)

Linear mixed-effects model fit by REML

  Data: Experiment_2_Data_Week_13


Random effects:

 Formula: ~1 | Plate_Number

        (Intercept)  Residual

StdDev:   0.3670628 0.1646617


Fixed effects:  Log_Sq_Average ~ relevel(Treatment, r = "Pink") + SVL_Change_1_13 +
Sex_Sq + SVL_Change_1_13:relevel(Treatment, r = "Pink")

 Correlation:

                                                      (Intr) rl(T,r="P")O rl(T,r="P")Y
SVL_C_ Sx_SqM r(T,r="P")O:

relevel(Treatment, r = "Pink")Orange                  -0.402

relevel(Treatment, r = "Pink")Yellow                  -0.415  0.345

SVL_Change_1_13                                       -0.683  0.568        0.582

Sex_SqM                                               -0.229  0.067        0.081
0.214

relevel(Treatment, r = "Pink")Orange:SVL_Change_1_13   0.391 -0.983       -0.330
-0.580 -0.075

relevel(Treatment, r = "Pink")Yellow:SVL_Change_1_13   0.416 -0.343       -0.982
-0.613 -0.087  0.346


Standardized Within-Group Residuals:

       Min         Q1        Med         Q3        Max

-1.9478788 -0.5176651 -0.1094772  0.5344634  2.3842235


Number of Observations: 61

Number of Groups: 4
```

#Linear mixed effects model of SVL_Change_1_13 and interaction with Log_Sq_Average and Treatment

```
SVL_lme = lme(Log_Sq_Average ~ SVL_Change_1_13 + relevel(Treatment, r = "Pink") + rele
vel(Treatment, r = "Pink"):SVL_Change_1_13, random = ~1|Plate_Number, data=Experiment_
2_Data_Week_13, na.action = na.exclude)

summary(SVL_lme)

anova(SVL_lme)


fig3=ggplot(Experiment_2_Data_Week_13, aes(x=SVL_Change_1_13, y=Log_Sq_Average, color
= interaction(Sex_Sq, Treatment))) +

  ylab("Log Telomere Length") +

  xlab("SVL change (g)") +

  ggtitle("SVL impact on early life telomeres") +

  geom_smooth(method = "lm", se = FALSE) +

  scale_color_manual(values = c("orange1", "orange3", "pink1","pink3", "yellow1","yell
ow3" ))

fig3
```