FINAL REPORT

South Carolina State Wildlife Grant SC-T-F15AF00731

South Carolina Department of Natural Resources

October 1, 2015 – September 30, 2018

Project Title: Potential Mechanism of Thermal Stress Impairment for Striped Bass Populations

Abstract: Striped bass (*Morone saxatilis*) populations have declined throughout their range, and prevailing causal hypotheses are associated with increased summer temperatures. Unlike the northern anadromous populations of striped bass that move into coastal estuarine systems in the summer, southern populations tend to move into freshwater habitat for thermal refugia (Scruggs 1957; Combs and Peltz 1982; Bjorgo et al. 2000). Because of the increased temperature associated with global climate change and extreme temperatures of shallow coastal systems, it is possible that coastal populations of striped bass are crowded into available temperature refugia (thermal niche squeeze) with detrimental results likely including direct mortality, decreased fecundity, and increased susceptibility to starvation, harvest, and parasites (Coutant 1985). We hypothesized that in addition to Coutant's (1985) individual-based impacts, thermal stress may result in evolutionary implications for striped bass populations, specifically through increased rates of telomere degradation. Telomeres are non-coding DNA regions composed of a conserved repetitive sequence that occur at the ends of linear chromosomes to protect the structural function of chromosomes as well as the DNA coding regions themselves. As the occurrence of organism-wide or critical tissue function loss can result in organismal death, increased net telomere loss rates have the potential to directly influence individual longevity (Barrett et al. 2013). Therefore, if thermal stress in striped bass results in higher telomere loss rates that are associated with decreased life spans, shifts in critical population life history traits (i.e. age and size at maturity, fecundity, etc.) would be expected.

Striped bass remain an important and popular recreational fish species in South Carolina and represent a key indicator species for coastal ecosystems. As such, SCDNR continues to be interested in proactively improving our understanding of the factors currently impacting striped bass populations in order to develop management strategies to address potential continued declines. Our mariculture and genetic research teams have leveraged their expertise in both finfish production/husbandry and molecular tool development in conducting manipulative laboratory experiments to determine if thermal stress levels experienced in SC coastal systems are capable of negatively influencing growth and telomere loss rates in striped bass. We initially hypothesized that thermal stress may result in evolutionary implications for striped bass populations, specifically through increased rates of telomere degradation. While potentially confounding factors in this study resulted in lower confidence in our comparisons of telomere length data between sampling time periods, we have demonstrated the significant effects that high temperatures and limited food have on striped bass growth and condition factor. Additionally, it appears striped bass may utilize regulation in telomerase activity to assist in reducing or minimizing impacts of potential stress sources that may increase telomere loss rates. Although our project results have not identified a clear mechanism of thermal stress impacts in striped bass, the results are contributing substantially to our understanding of appropriate protocols and tools to evaluate telomere changes, such as incorporation of individual tracking and telomerase quantification with avoidance of short cut qPCR techniques, as well as increasing our knowledge of the complexity of cell replication processes and the influences of

environmental factors on those processes. As average annual temperatures within the native striped bass range increase, we may expect to see an accelerated decrease in total area of thermal refugia for striped bass during the summer months thereby presenting habitat constraint challenges for the already declining wild populations.

Purpose and Objectives- Our specific project objective was to determine if thermal stress associated with climate change increases the rate of telomere loss, thus decreasing southern striped bass population fitness. Our project goal was intended to be achieved over a two-year period with efforts in the first year focusing on the generation of telomere data, which was to be completed during the second year along with all data analyses and interpretation. Our specific project objectives for Year 1 included:

(i) Telomere detection and data acquisition of samples from the initial sample period.

(ii) Telomere detection and data acquisition of samples from experimental sample period 2.

(iii)Telomere detection and data acquisition of samples from experimental sample period 3.

During Year 1, we determined that the decision to recommend the continuation of this project would be more informed if our Year 1 results demonstrated telomere length changes within our study period. If telomere length changes were to occur, we expected the most drastic differences between the initial sampling event and the final sampling event. Therefore, we adjusted our telomere detection and data acquisitions objectives to focus on the initial sampling period and final experimental sampling period 4. In addition to adjusting project workflows for telomere length analyses, we also added an additional component to this project that will allow us to track individual fish telomere lengths through each time period.

The adjustments in Year 1 objectives directed adjustments to our objectives for Year 2. Our goal for Year 2 was to complete telomere length analyses for experimental sample periods 2 and 3. However, during Year 2, the proprietary analysis supply kits for telomere length were unavailable from the supplier for an extended period. Since the date the product would return to production was unknown, we focused time during Year 2 investigating an alternative method for assessing telomere length utilizing a more time efficient qPCR methodology. Unfortunately, our experimental results were neither reproducible nor reflective of patterns documented with the validated TRF methodology. In accordance with our approved no cost extension due to the supply availability, we returned to processing samples using the TRF methodology once kit availability resumed, and the remaining revised tasks completed during Year 3 included:

(iv)Telomere detection and data acquisition of samples from sample period 2.

(v) Telomere detection and data acquisition of samples from sample period 3

(vi) Estimate of telomere lengths from all samples.

(vii) Data analyses to determine influence of thermal stress on telomere loss rates.

(viii) Integration of telomere and metabolic results.

(ix) Compile project results to present to the scientific community and fishery managers.

qPCR Methodology Experiment:

Accomplishments:

During the second year of our current project, we adjusted our telomere length data acquisition workflow to focus on an alternative method for estimating telomere length because the proprietary materials for the TRF methods used during Year 1 became unavailable through the supplier. With the production date of these products being unknown, we evaluated an alternative method of telomere length estimation using quantitative PCR (qPCR). While the TRF method is considered the 'gold standard' of telomere length assays, the qPCR method is preferred by many researchers because it is fast, cheap, and can be performed in any lab with a qPCR machine. The qPCR method quantifies the amount of telomere DNA (T) in a sample relative to the amount of a standard control gene (S) (originally developed by Cawthon 2002). Cawthon suggests that the relative amount of telomere DNA (T/S ratio) is a proxy for telomere length, and in his study, compared the novel qPCR assay for telomere length estimation to the TRF Southern blotting method (Figure 1).

Since 2002, Cawthon developed (or has been an author on) two other methods using qPCR to estimate telomere length. These methods use the same T/S ratio as a proxy for telomere length but differ from the original paper by changing primer sequences and PCR cycling conditions. We evaluated all three primer sets and PCR cycling parameter sets in our lab and selected the one with the best PCR efficiency (Figure 2). We also tested three control genes, 18S, GADPH, and L9, which have been used specifically for striped bass in other studies. We selected 18S based on PCR efficiency (Figure 3).

After optimization, we assayed the same DNA samples that were evaluated for telomere length during Year 1 (initial and final sampling periods) using the TRF method with the qPCR method. DNA isolations were standardized to 10 ng/µL for template in qPCR assays. All samples were amplified with both telomere (T) primers and control gene (S) primers in triplicate, and the average critical qPCR values (Cq) were used to estimate target DNA masses. The standard curves of T and S (Figures 2 and 3, respectively) were used to extrapolate the amount of target DNA in each sample (i.e. the amount of telomere DNA or control gene DNA in 10 ng of genomic DNA). Target DNA quantities were used to calculate the T/S ratio for each sample.

Our results for the qPCR assays were unexpected. We found no relationship between the TRF data from Year 1 and the T/S ratio produced with qPCR (Figure 4). Furthermore, we found changes in both the control gene and the telomere qPCR between the initial and final time periods, suggesting that 18S may not be an appropriate control gene. However, because the T/S ratio was conceived for relative comparisons of telomere length of between individuals, we argue that single copy control genes are not necessary when comparing telomere lengths by qPCR of two groups of individuals (i.e. control and stressed groups). Therefore, we evaluated the telomere qPCR data in relation to the Year 1 TRF data independently. Following this approach, we again found no relationship between the quantity of telomere DNA and the TRF data collected in Year 1 (Figure 5).

To further investigate the feasibly of using qPCR telomere data for this project, we plotted both the T/S ratio and the raw telomere quantification data against changes in condition factor to determine if the same patterns of less variability and overall telomere length loss in the control group over time that we saw in the TRF data (Figure 6) could be found using qPCR. Surprisingly, given the discordance in qPCR data and TRF length, we found a similar pattern between the qPCR and the TRF data when only using

the raw telomere quantification data in that the amount of variation within groups differed substantially. As with the TRF data, variation surrounding the average change in Cq was lower in the control group (-0.02 ± 0.90) relative to the stressed (0.29 ± 2.27) (Figure 7); however, these data fail to recover the pattern of overall loss of telomere DNA in the control group. The pattern of lower variation in the control group was not evident in the scatter plot of T/S ratio and change in condition factor (Figure 8). While the raw telomere quantification data seem to show a similar pattern to the TRF data collected in Year 1 with respect to variation in control and stressed groups, we were not comfortable moving forward with qPCR data acquisition following these methods due to the lack of a relationship between telomere qPCR data and average telomere length obtained from the TRF method.

Towards the end of Year 2, the proprietary materials for the TRF analysis again became available and we received approval for a one-year, no-cost extension to complete the project as originally planned using TRF analysis.

Significant deviations:

During Year 2, the proprietary analysis supply kits for telomere length were unavailable from the supplier for an extended period. Since the date the product would return to production was unknown, we investigated an alternative method for assessing telomere length using qPCR. In addition to delays from lack of material for TRF analysis, our adjusted Year 1 objectives carried over into Years 2 and 3.

Terminal Restriction Fragment (TRF) Evaluations:

Accomplishments:

NOTE: Grant SC-T-F15AF00731 utilizes samples and methodology from a previous study (2009). At the time, funding was not available to process these samples and thus are now analyzed in this current study.

During 2009, we conducted a robust repeated measures experimental design to evaluate influences of thermal stress on striped bass; a simplified schematic of the design is shown in Figure 9. Excess hatchery fish from the 2007 production year (~2 years of age), that had been maintained in a single pond at the Dennis Wildlife Center until transfer to the Marine Resources Research Institute, were used in the experiments so that differential environmental experiences or resource exposures would not confound any observed treatment effects. We utilized three replicate systems for thermally stressed and control treatment groups. Thermally stressed treatments were maintained at 28°C following a gradual temperature increase from 22°C over a week; control groups were maintained at 22°C. Experimental temperatures were determined based on evaluation of local long-term water temperature data from the Cooper River. Individuals were randomly assigned to treatments and replicate systems within treatments (n=20 fish/system, 120 total fish). Prior to the start of the experiment—and at all sampling periods—all individuals were weighed (g) and measured (mm TL), and a tissue sample was removed from the anal fin. Each tissue sample was subdivided and one part preserved in 95% EtOH and the other in sarcosyl urea.

All systems were maintained on flow-through water of ~7 ppt throughout the duration of the experiment, and standard husbandry protocols were used for monitoring and maintenance of the water quality in the experimental systems. The experiment began on February 11, with the first intermediate sampling occurring on April 29, the second sampling on July 29, and the final sampling completed on

September

25. During the first experimental period (Feb-Apr), food was provided *ad libitum* to all treatments (with consumption levels recorded); during the rest of the experiment, food availability was standardized across systems with consumption levels still recorded. Following the first experimental period (Feb- Apr), treatments (i.e. temperatures) were switched among groups for the remainder of the experiment (Apr-Sept) with gradual acclimation to treatment temperatures occurring during a transition week.

During Year 1, we adjusted our telomere length data acquisition workflow to focus on the initial and final sampling periods to identify the potential maximum effect of thermal stress on telomere length. DNA was isolated from fin tissue using the Promega SV Wizard DNA isolation kit in a final volume of 80 µl of ultrapure water. DNA was quantified using a Qubit fluorometer and 1 µg of DNA was added to the Roche TeloTTAGGG Telomere Length Assay kit for all telomere length assays. The TeloTTAGGG Telomere Length Assay kit follows a standard terminal restriction fragment (TRF) length analysis, where genomic DNA is exhaustedly digested with a cocktail of high-cut frequency restriction endonucleases leaving only telomere and sub-telomeric sequences intact, then the remaining telomere fragments are separated by size using gel electrophoresis, followed by a Southern transfer to a positively charged nylon membrane and subsequent chemiluminescent detection. Our laboratory protocols followed the kit manufacture's recommendations with the exception of the gel electrophoresis methods. Instead, we employed pulsed-field gel electrophoresis, where DNA is 'zig-zagged' through the gel matrix by alternating the angle of applied electric current. The 'zig-zagging' effect allow smaller DNA fragment to pass by larger DNA fragments during the separation without the potential blocking effect that larger molecules present. Ultimately, pulsed-field electrophoresis allows for better resolution of a wide range of fragment sizes within a single gel concentration. We used 0.8% megabase agarose gels run at 5V/cm with switch times from 0.5 – 7.0 seconds at 14º C for 5 hours.

Following the TeloTTAGGG Telomere Length Assay kit's protocols, prior to Southern transfer, DNA was nicked with HCL to increase transfer efficiency and denatured in the gel to expose all telomere probe hybridization sites. Southern transfers were performed with Whatman's Turbo blotter systems and transfers were run overnight. DIG-labeled telomere probes were hybridized with telomere sequences that were fixed to positively charged nylon membranes, and unbound and non-specifically bound probes were washed away during stringent washes. Telomere sequences were detected with the chemiluminescent reaction of *CDP-Star* and alkaline phosphatase coupled with a DIG specific antibody.

Chemiluminescent signal from the Southern blots were imaged with a Syngene G:Box imaging system (Syngene) and TRF data was acquired using the GeneTools software (Syngene). Background noise associated with non-specific hybridization of the telomere probe was subtracted, molecular weight size standards were checked for correct assignment, and calibration curves were created automatically for each size standard lane using a log-linear model. Grayscale profiles of signal intensity at each molecular weight position on the gel lane were acquired for all samples. All resulting intensity at molecular weight data was exported directly to Excel. To correct for increased signal intensity due to multiple telomere probe hybridizations in longer telomeric segments, signal intensity was divided by the molecular weight at each position on the blot image; the resulting corrected value (C_i) was used in all final analyses. Average TRF was calculated using the following formula:

$$\overline{TRF} = \sum(OD_i)/\sum(C_i)$$

An additional component added to our Year 1 Objectives was to genetically identify each fish to enable us to evaluate an individual's telomere length changes in response to thermal stress and in relation to individual fish growth. Each DNA isolation was genotyped at 12 polymorphic microsatellite loci and identity analysis was conducted on all genotypic data. Genotyping protocols followed the standard operating procedures of SCDNR's striped bass genetics research program. All genotyping attempts for DNA isolations for this project have been successful. In addition to allowing for individual identification, microsatellite genotyping allows us to verify that DNA samples were not cross-contaminated with each other prior to telomere length assays.

During Year 3, we completed telomere length assays using the TRF methods for all of the remaining samples (n=203, 26 Southern blots), resulting in telomere length data for 375 individuals (48 Southern blots). In addition, all remaining samples were genotyped at 12 microsatellite loci for genetic identity analysis to track individual fish throughout the duration of the experiment. Once the final dataset was compiled, average telomere length for each fish was plotted in line charts specific for each tank and treatment. Average total lengths, weights, condition factors, and telomere lengths were calculated for each tank system and t-tests were performed for the control and stressed groups at each time point using Sigma Stat software.

After the first time period, when fish were fed to satiation, there were no differences in growth between the control and stressed treatment groups. These results were unexpected based on condition factors of wild fish in local systems at these temperatures. Therefore, for the remainder of the experiment food was limited to a standardized mass for each tank in order to mimic a more likely scenario that striped bass might experience in the wild. After food was limited, significant differences in weight and condition factor were observed (Figure 10) at both Sampling Times between the control and stressed treatment groups ($p=0.04$-0.005). There were no significant differences in total length or telomere length between the treatment groups at any of the four Sampling Times ($p=0.092$-0.893, Figure 10). However, we observed a concerning pattern in the telomere length data that stood out as a potentially confounding factor. A scatter plot of telomere length verses Southern Blot date (Figure 11) suggest two distinct clusters of telomere lengths, which appear to be associated with the manufacturer's hold on the proprietary supply kits for the telomere length assays. Specifically, samples run before the end of Jan 2017 (prior to the production availability) in general show shorter telomere lengths than those processed after Jan 2017 (after production availability resumed). Therefore, we are presenting our final results with caution and further work will be needed to determine if these concerns are warranted.

Our preliminary results from Year 1 included two of the six control tanks and all three of the stressed tanks for Sampling Times 1 and 4 (T1 and T4, respectively). Although not included in the Year 1 preliminary analyses, an additional nine samples were processed from the control group at Sampling Time 1 (i.e. most fish from a 3rd control tank). The corresponding Sampling Time 4 samples for this control tank were not processed in Year 1 because of time constraints due to the addition of our objective to genotype all samples for identity analysis. Therefore, at the beginning of Year 2 (Jan 2017), we proceeded with telomere length assays using the remainder of the telomere length assay kit supplies that were purchased at the beginning of the project, which was enough for eight samples. As described above, during Year 2 the supply kits became unavailable by the manufacture for ~9 months. Once available again, all remaining samples were processed with supply kits purchased in October 2017. Because all of the Sampling Time 1 and 4 samples for our stressed treatment group were processed with original supply kits, comparisons between four of our control and all stressed treatment groups at Sampling Time 1 and 4 may be compromised.

Comparisons between treatments were not confounded by supply kit effects for all Sampling Time 2 and 3 samples because all were processed with supply kits purchased after production resumed in 2017. A general pattern of increasing telomere length was observed in four of the six control tanks between Sampling Time 2 and 3, while the remaining two control tanks showed an overall decreasing telomere length pattern. Fish in all three stressed treatment tanks exhibited the similar pattern of telomere change with minor gains and losses within individuals but no overall trend of lengthening or shortening. When the two control tanks showing the opposite pattern of the other four are removed from the analysis, significant difference in telomere length are observed at Sampling Time 3 between the control and stressed treatment groups ($p=0.042$, Figure 12). The mechanism driving the unique pattern of telomere loss for fish in these two tanks is unclear. However, one possibility is that these fish experienced a different environment than all other control fish during the first experimental period as these tanks were housed within the same experimental system and the third tank within the system housed a control group only for the second and third experimental periods.

These results suggest that thermal stress in conjunction with food limitation certainly has an effect on striped bass growth. Scatter plots of grow rates based on weight and condition factor verses telomere length changes all show distinct groupings of control and stressed treatment fish (Figures 13 & 14) along the axis depicting changes in growth (x-axis). In contrast, overlap between the stressed and control treatment groups along the axis depicting telomere length change (y-axis) suggest within the timeframe of our experiment, thermal stress with food limitation likely has less of an impact on overall telomere length. However, we are cautious to draw conclusions on the mechanism of how these stresses influence telomere length given that we observed potential supply kit effects and potential tank effects.

We initially hypothesized that thermal stress may result in evolutionary implications for striped bass populations, specifically through increased rates of telomere degradation. While potentially confounding factors in this study resulted in lower confidence in our comparisons of telomere length data between Sampling Times, we have demonstrated the significant effects that high temperatures and limited food have on striped bass growth and condition factor. Additionally, it appears striped bass may utilize regulation in telomerase activity to assist in reducing or minimizing impacts of potential stress sources that may increase telomere loss rates. Although our project results have not identified a clear mechanism of thermal stress impacts in striped bass, the results are contributing substantially to our understanding of appropriate protocols and tools to evaluate telomere changes, such as incorporation of individual tracking and telomerase quantification with avoidance of short cut qPCR techniques, as well as increasing our knowledge of the complexity of cell replication processes and the influences of environmental factors on those processes. As average annual temperatures within the native striped bass range increase, we may expect to see an accelerated decrease in total area of thermal refugia for striped bass during the summer months, thereby presenting habitat constraint challenges for the already declining wild populations.

Significant deviations:

During Year 1, we determined that the decision on recommending the continuation of this project would be more informed if our Year 1 results demonstrated telomere length changes within our study period. If telomere length changes were to occur, we expected the most drastic differences between the initial sampling event and the final sampling event. Therefore, we adjusted our telomere detection and data acquisitions objectives to focus on the initial sampling period and experimental sampling period 4. In addition to adjusting project workflows for telomere length analyses, we also added an additional

component to this project that allowed us to track individual fish telomere lengths through each time period.

Literature Cited:

Bjorgo, K., J. Isely and C. Thomason. 2000. Seasonal movement and habitat use by striped bass in the Combahee River, South Carolina. Transactions of the American Fisheries Society 29: 1281–1287.

Barrett, E., A. Burke, M. Hammers, J. Komdeur, and D. Richardson. 2013. Telomere length and dynamics predict mortality in a wild longitudinal study. Molecular Ecology 22: 249-259.

Cawthon, R. 2002. Telomere measurement by quantitative PCR. Nucleic Acids Research 30(10): e47.

Combs, K. and L. Peltz. 1982. Seasonal distribution of striped bass in Keystone Reservoir, Oklahoma. North American Journal of Fisheries Management 2: 66-73.

Coutant, C. 1985. Striped bass, temperature, and dissolved oxygen: a speculative hypothesis for environmental risk. Transactions of the American Fisheries Society 113: 666-671.

Scruggs, G. 1957. Reproduction of resident striped bass in Santee-Cooper reservoir, South Carolina. Transactions of the American Fisheries Society 85: 144-159.

Estimated Federal Cost: The remainder of the project funds were expended during the final project period; total federal cost was $111,250.

Recommendations: Close the grant.

Prepared by: Matt Walker; Tanya Darden – 18 Dec 2018

Figures:

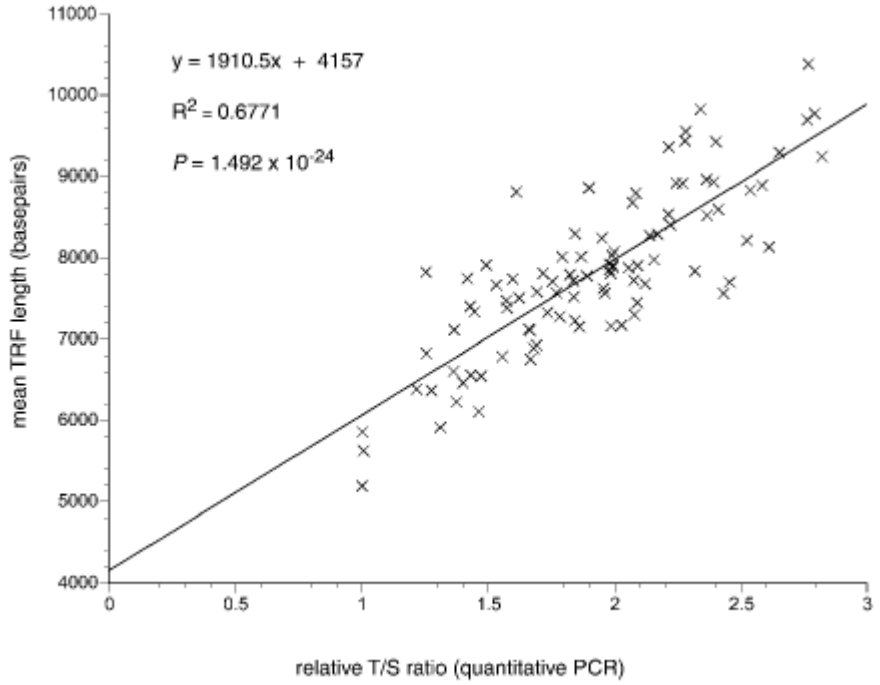

Figure 1. Cawthon 2002: relationship between T/S ratio and average TRF length.

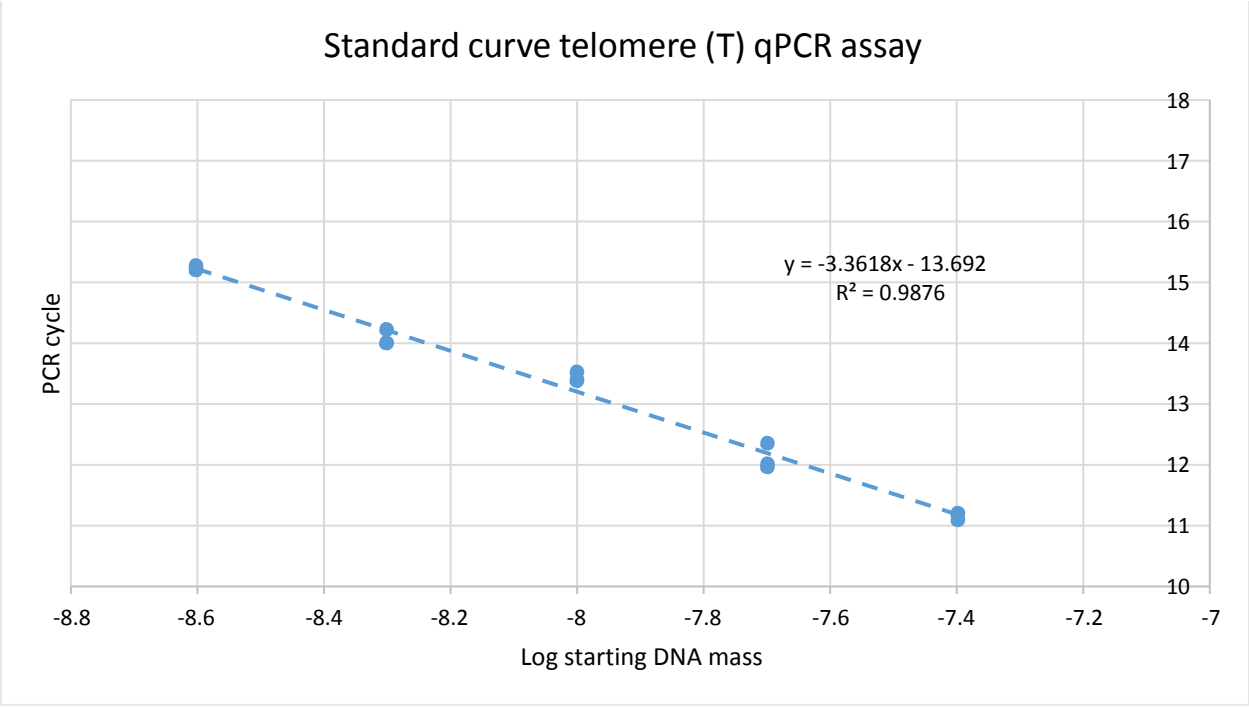

Figure 2. Standard curve for telomere qPCR assay for dilution series of striped bass DNA.

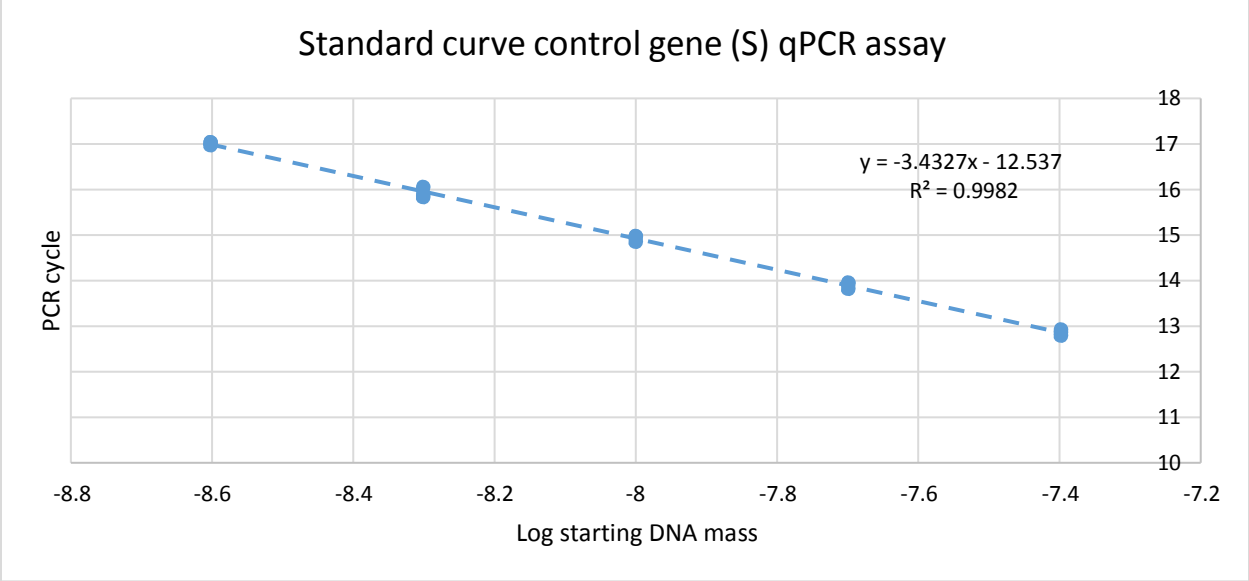

Figure 3. Standard curve for control gene qPCR assay for dilution series of striped bass DNA.

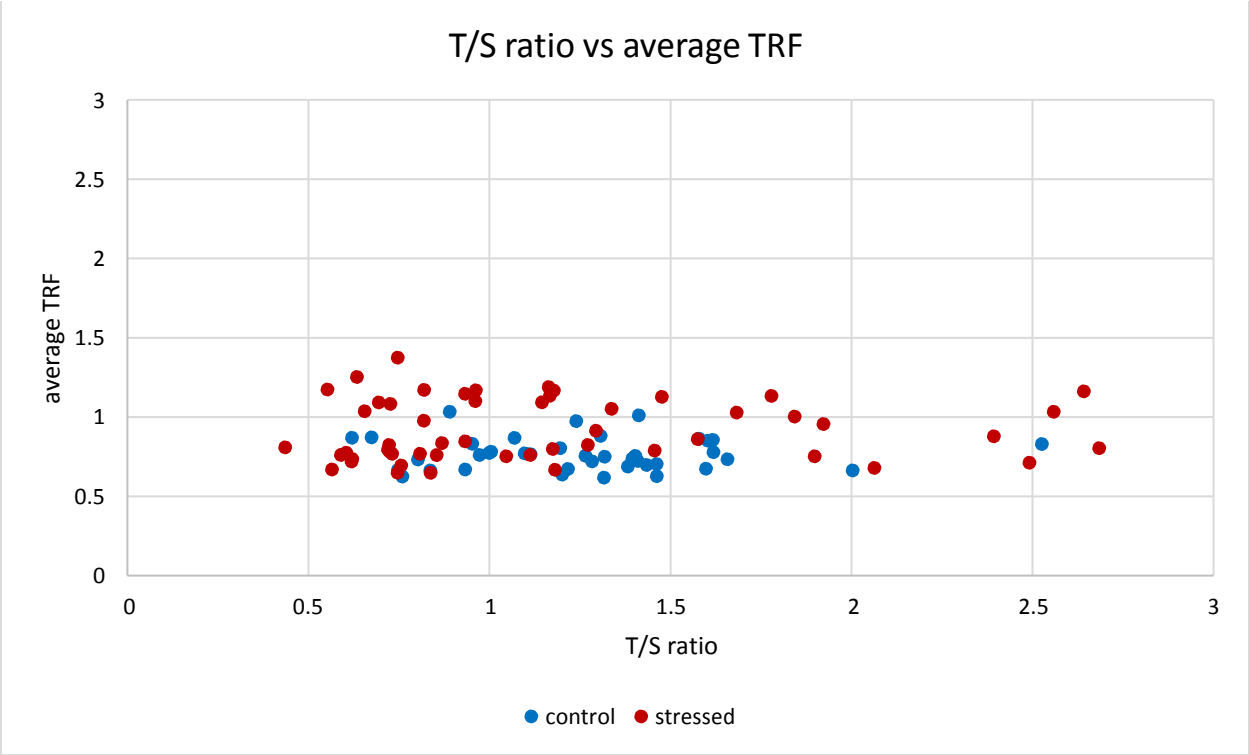

Figure 4. Scatter plot showing relationship between T/S qPCR values (Cq) and average TRF.

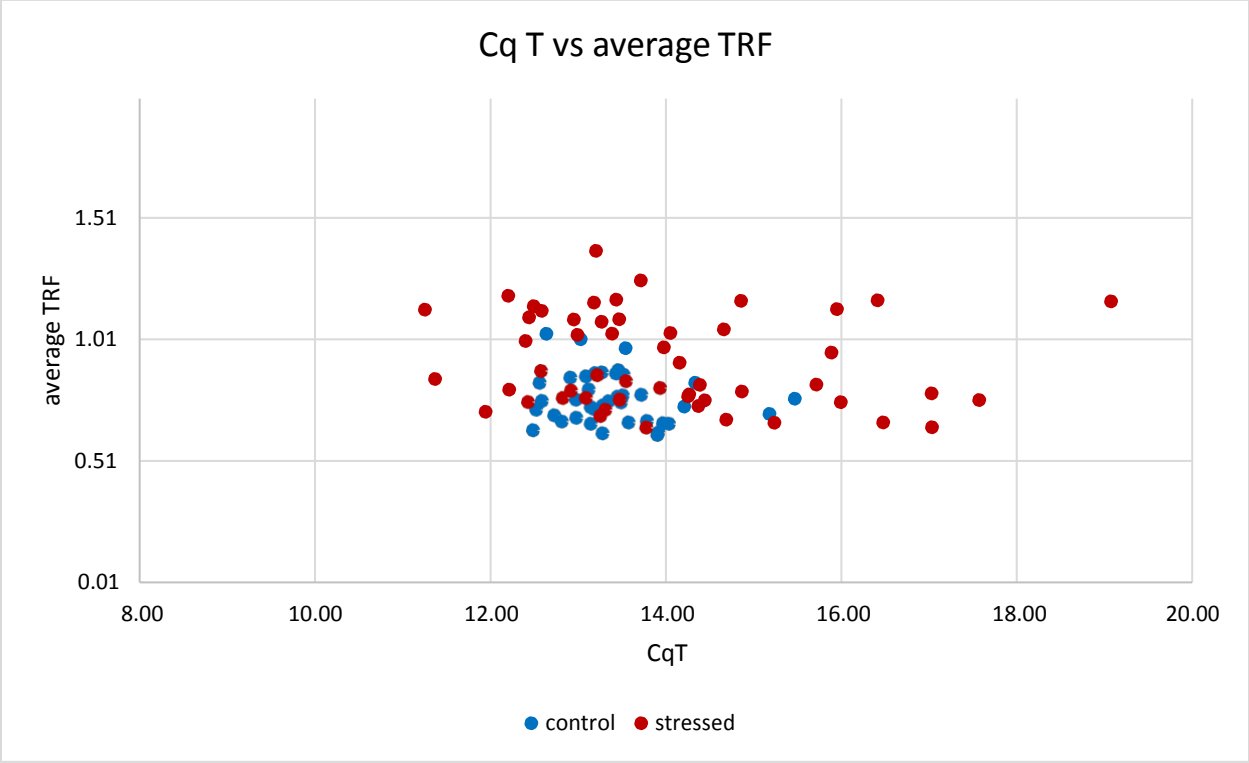

Figure 5. Scatter plot showing relationship between the raw telomere qPCR values (Cq) and average TRF.

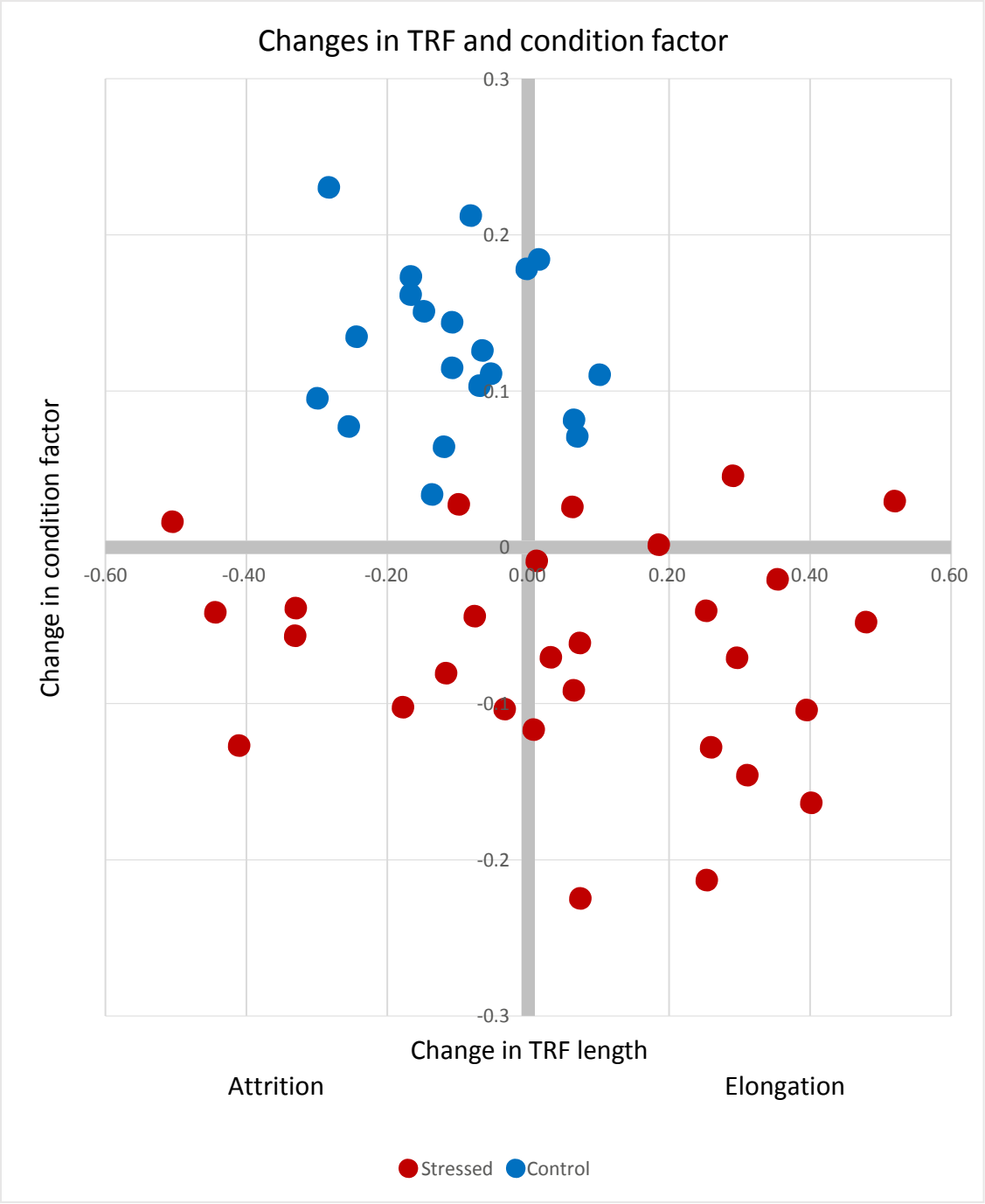

Figure 6. Scatter plot showing the relationship between changes in telomere length and changes in condition factor.

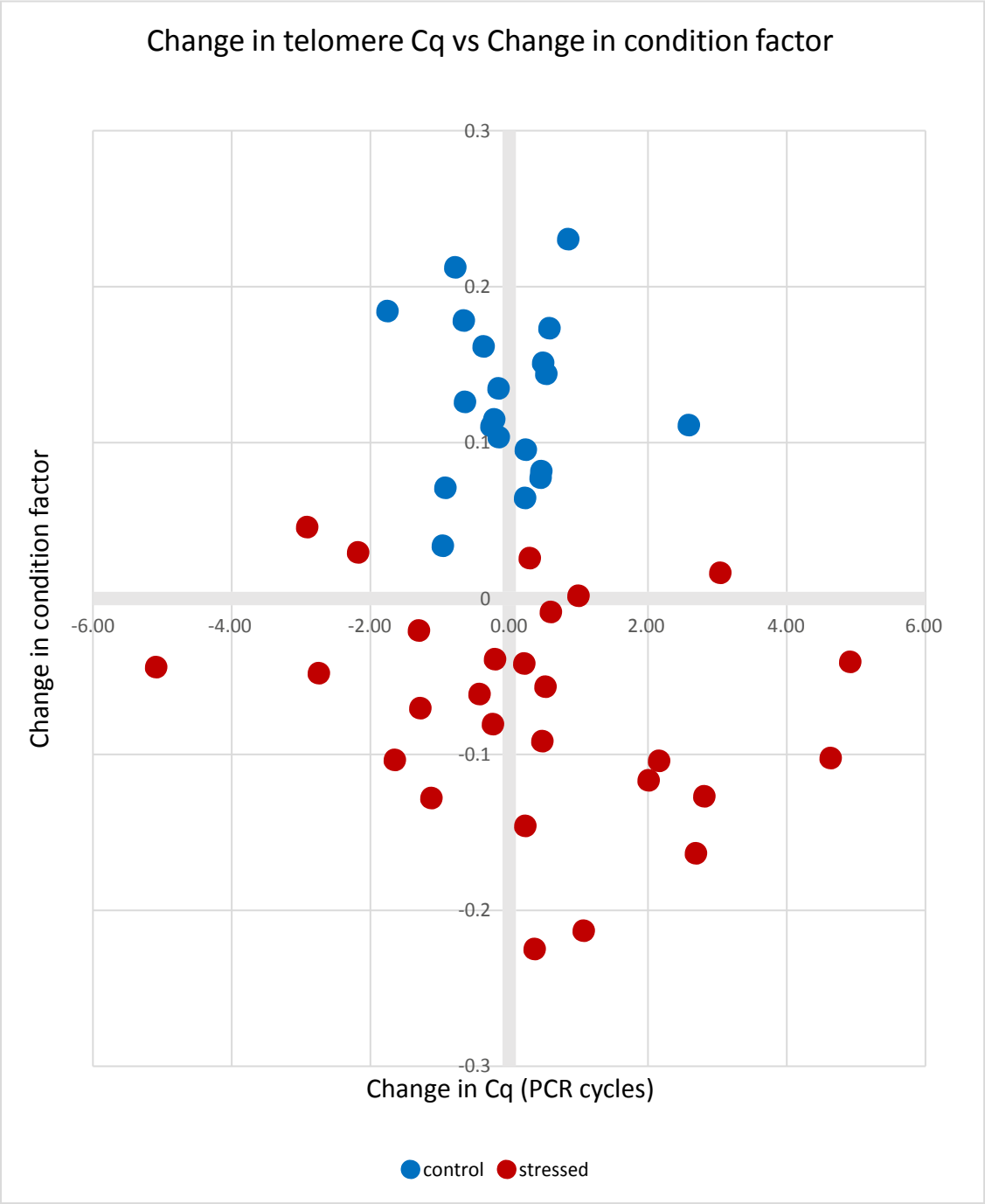

Figure 7. Scatter plot showing the relationship between changes in qPCR vaules and changes in condition factor.

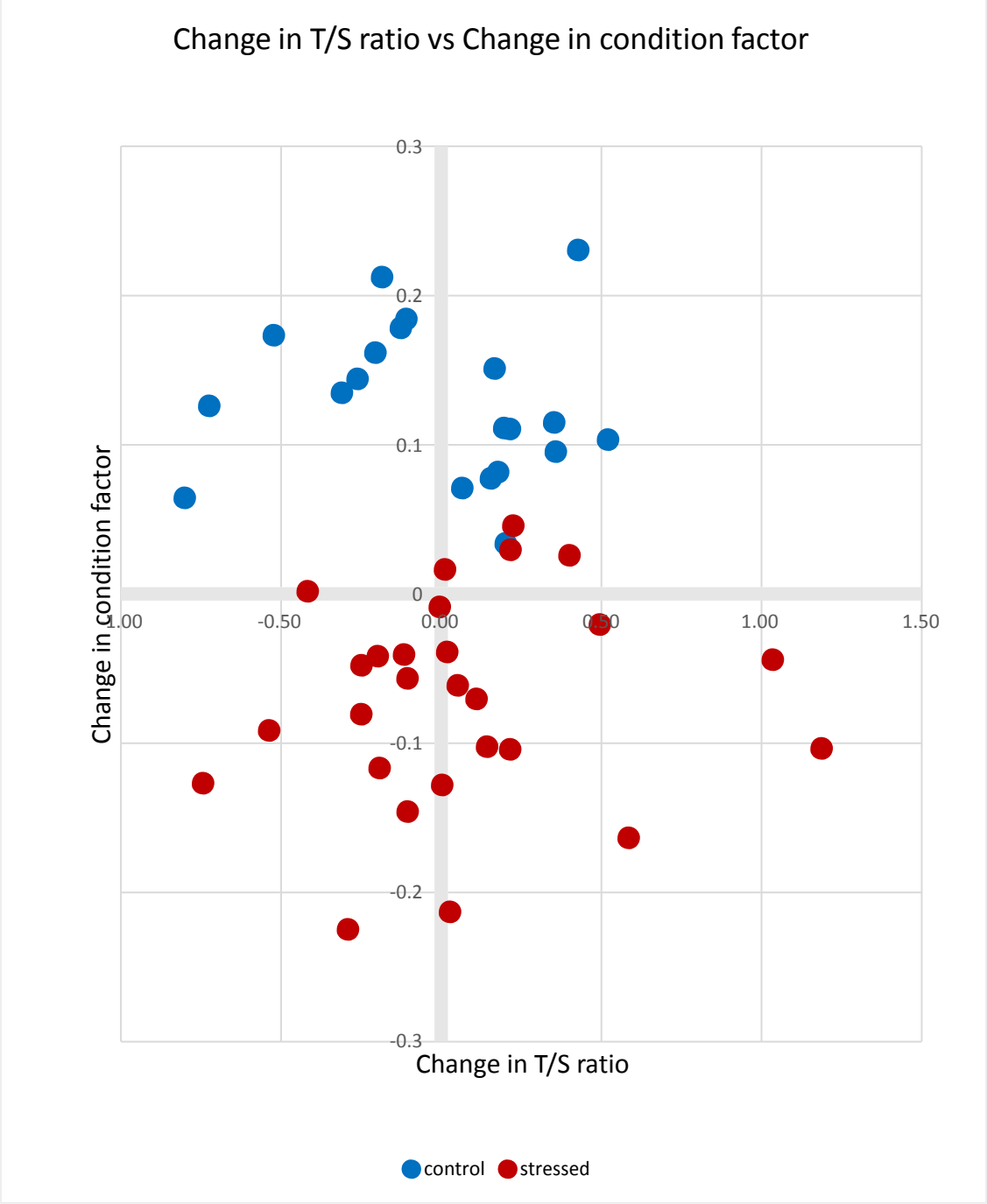

Figure 8. Scatter plot showing the relationship between changes in T/S vaules and changes in condition factor.

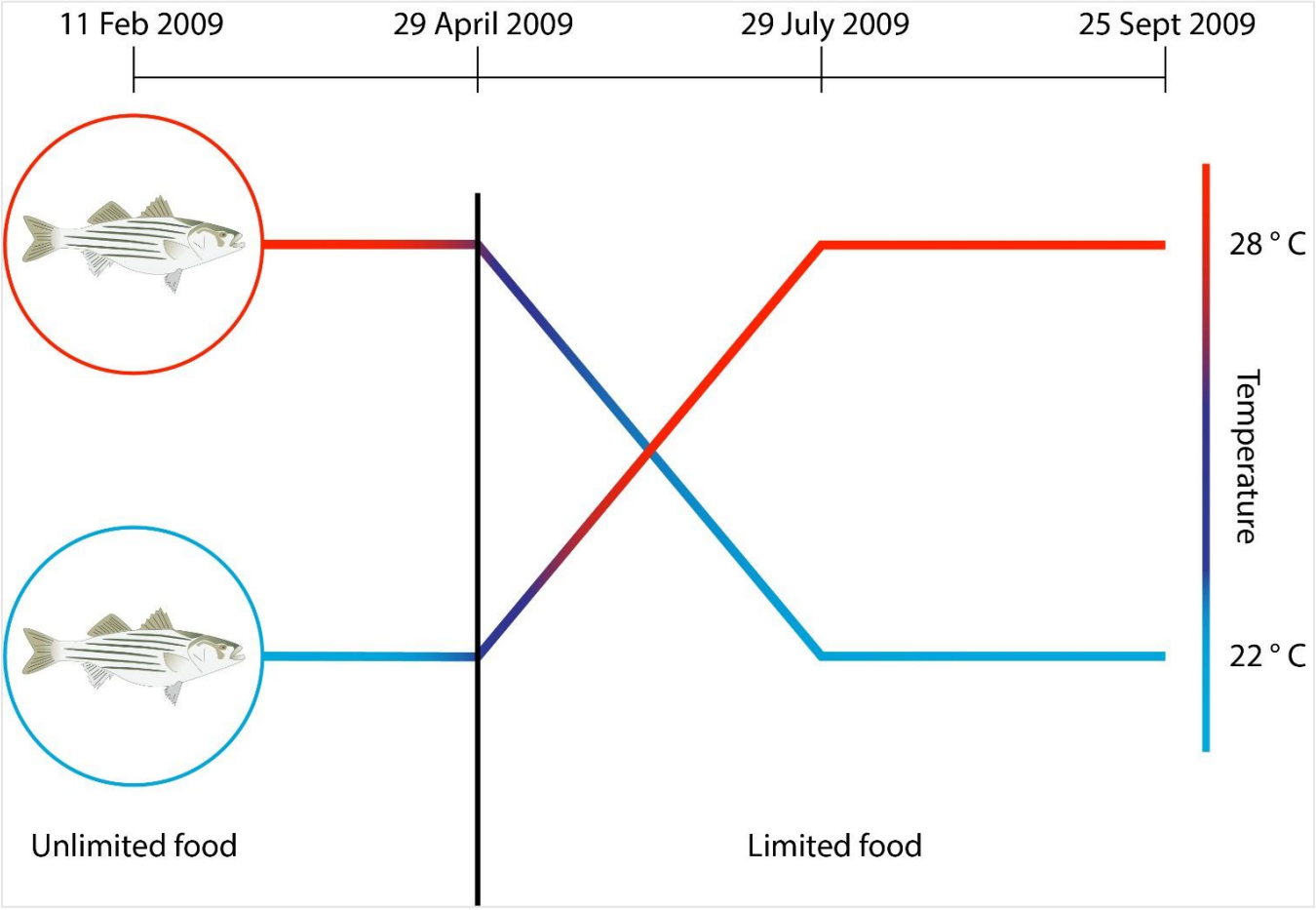

Figure 9. Simplified schematic of the design showing temperature changes, food availability and sampling dates. The vertical black line represents the time where treatments were switched and food availability was limited.

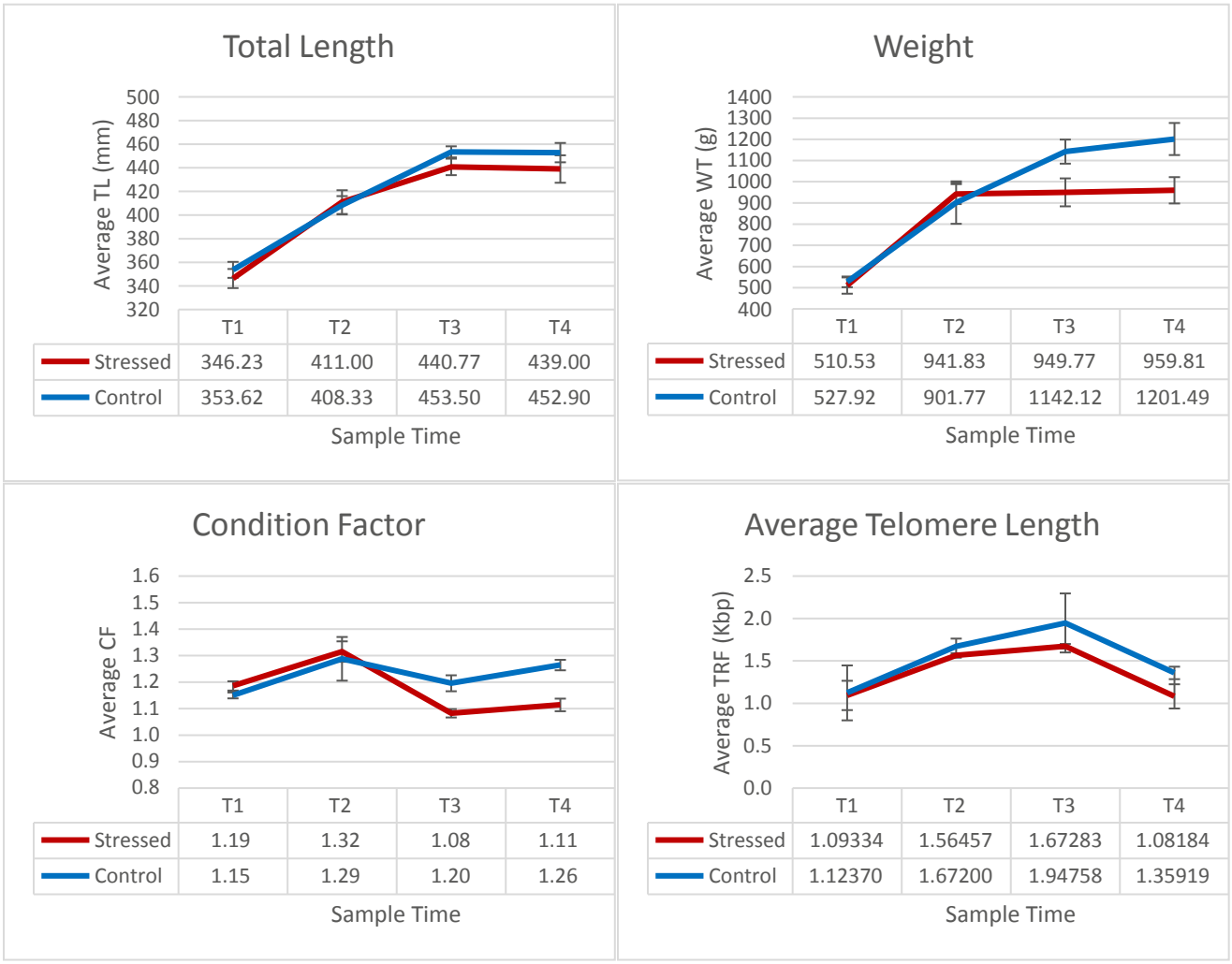

Figure 10. Line charts of average total length, average weight, average condition factor and average telomere length at each Sampling Time with tables containing corresponding values.

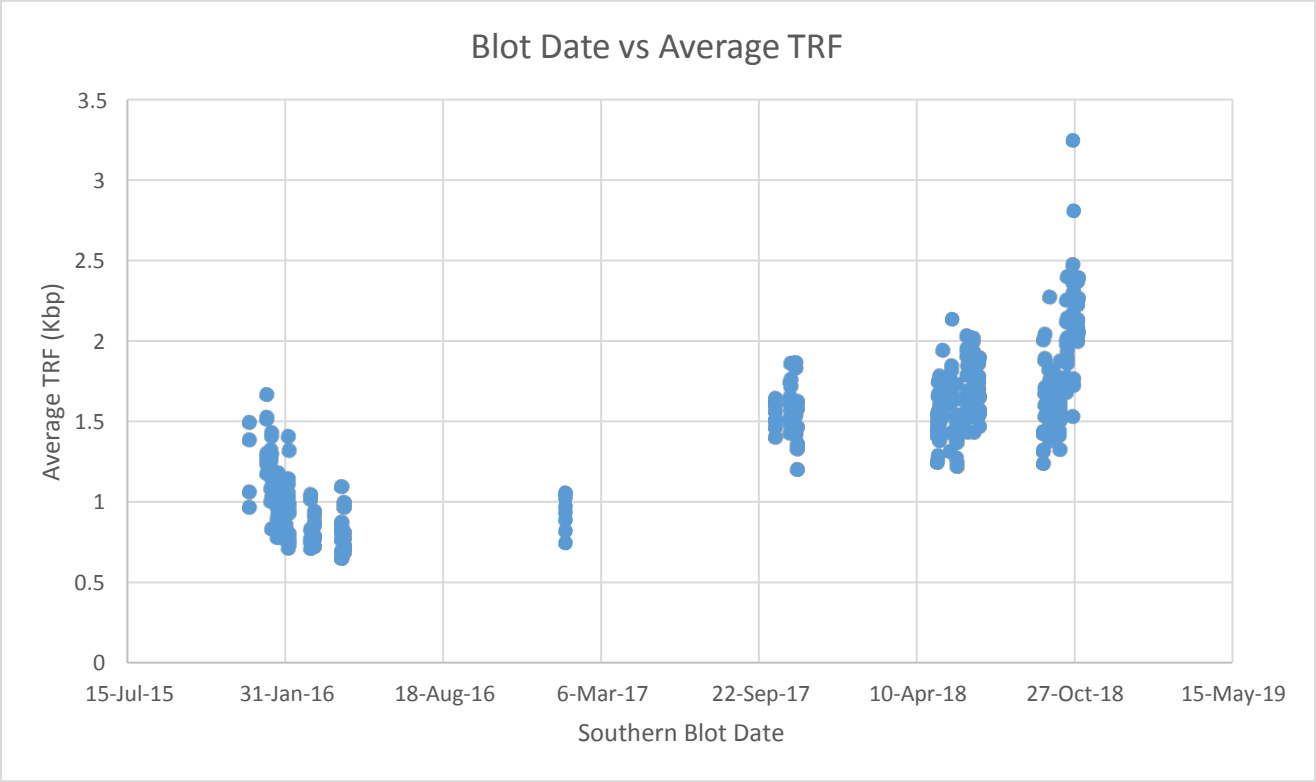

Figure 11. Scatter plot of Southern blot date verses average telomere length.

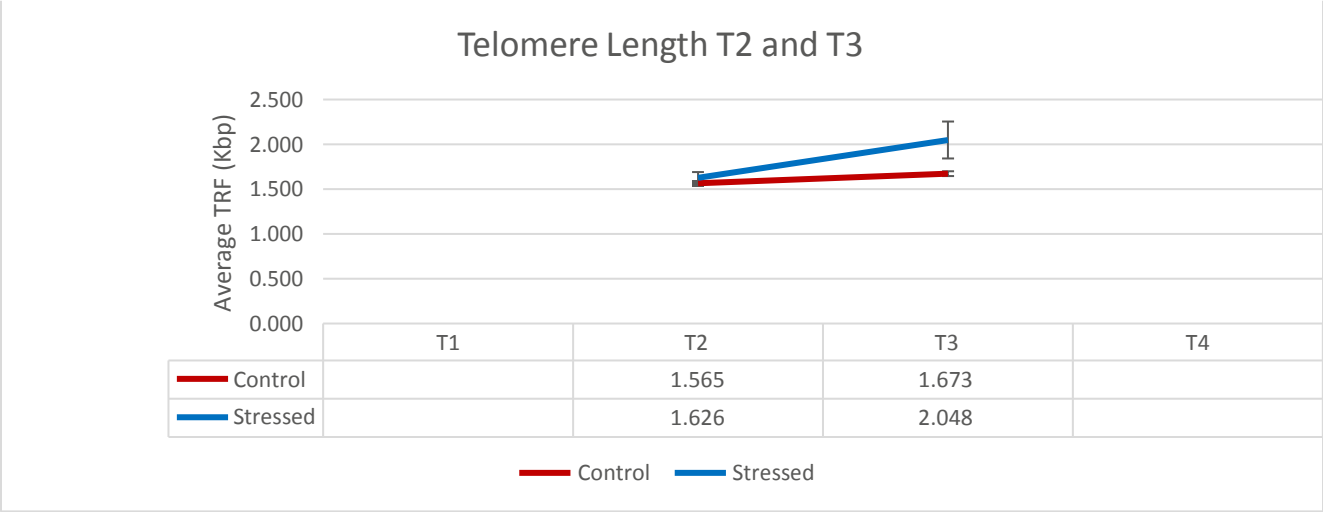

Figure 12. Line chart of average telomere length excluding two control tanks, which showed the opposite pattern of all other control tanks.

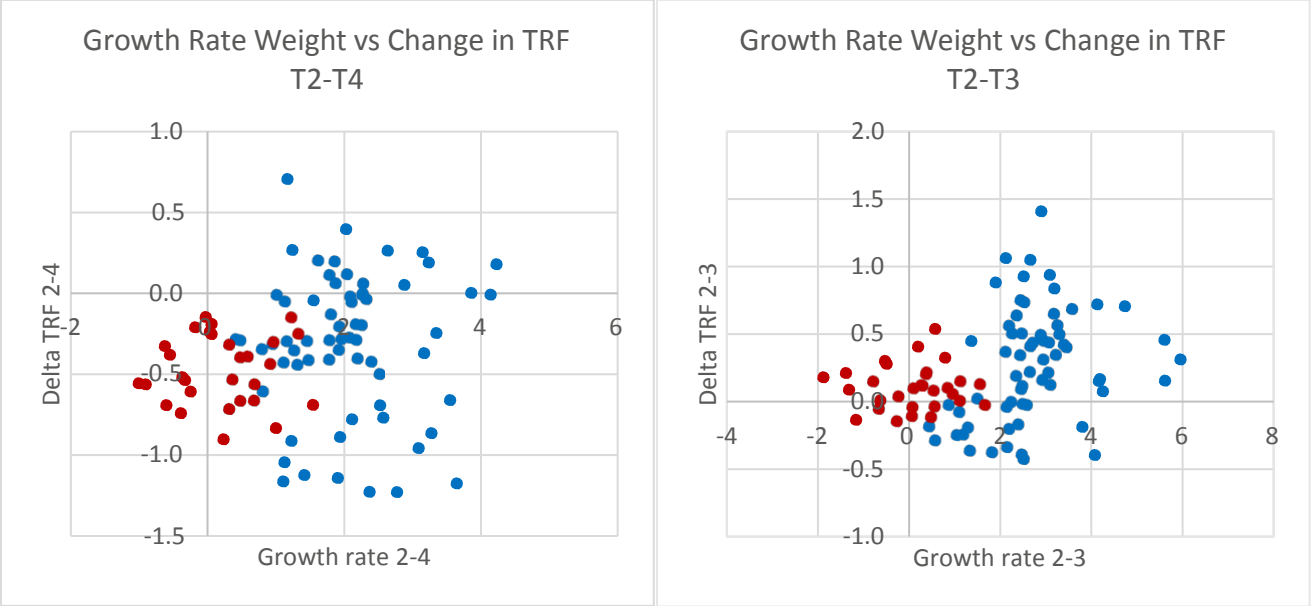

Figure 13. Scatter plot of growth rate (based on weight) verses change in telomere length for Sampling Times 2-4 (left) and 2-3 (right).

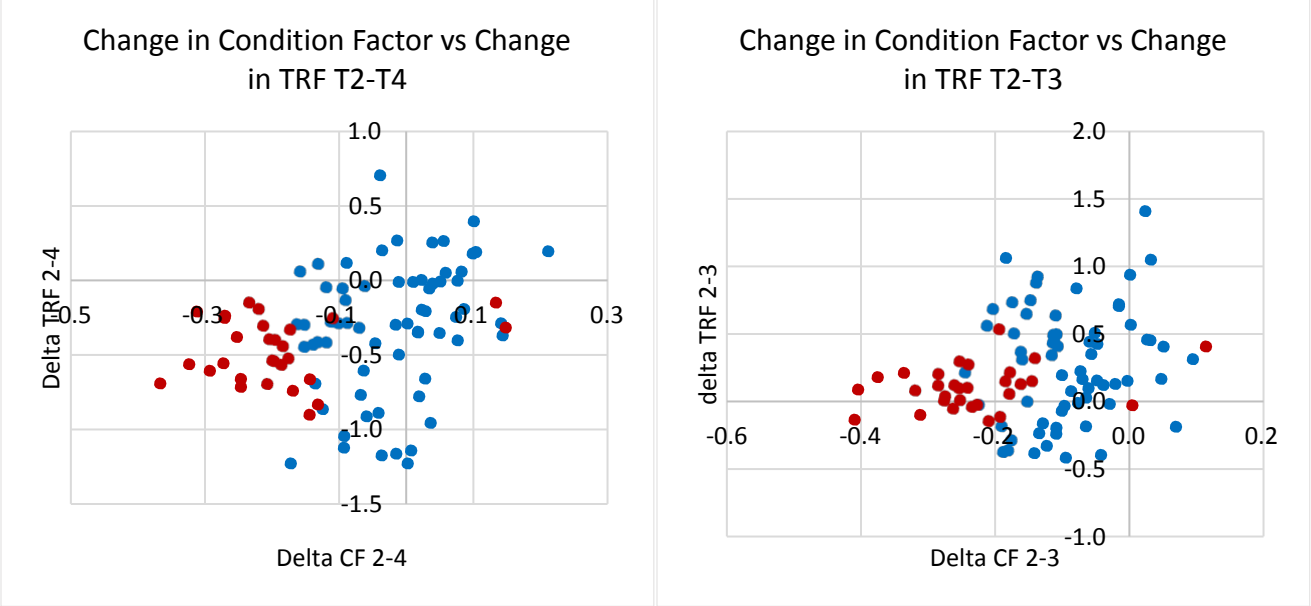

Figure 14. Scatter plot of change in condition factor verses change in telomere length for Sampling Times 2-4 (left) and 2-3 (right).