

Severe inbreeding of zebrafish results in a shortened lifespan independently of telomere length

Paolo Turano¹, Yukiko Imai², Toshihiro Kawasaki^{2,3}, Noriyoshi Sakai^{2,3}

¹Department of Microbiology and Molecular Genetics, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ, USA

²Department of Gene Function and Phenomic, National Institute of Genetics, Mishima, Japan

³Department of Genetics, School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Mishima, Japan

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Abstract

Model organisms have allowed researchers to gain knowledge on mechanisms that would have otherwise been nearly impossible to obtain; as such, they are a critical asset to researchers alike. *Danio rerio*, also known as zebrafish, have become increasingly popular due to their utility in life sciences, yet the high degree of genetic heterozygosity is a major shortcoming of the model. In recent years, Dr. Sakai and his lab have developed a highly genetically homozygous strain of zebrafish via inbreeding in order to better the model. This inbreeding however resulted in a significant decrease in their lifespan for reasons that are poorly understood. Here, we attempted to identify the cause of this decrease in lifespan by measuring the telomere length of both the commonly used AB and newly developed inbred strain, ABM, via quantitative polymerase chain reaction, qPCR. We found no significant difference in telomere length 5 days

post fertilization. This work suggests telomeres do not play a role in the premature death of the highly homozygous inbred strain ABM.

Introduction

The usage of model organisms has proven to be an invaluable tool in biomedical sciences. Indeed, there is little doubt the scientific community would have been able to make such rapid advancements without them. In the past century or so, model organisms have allowed researchers to elucidate the mechanisms of action behind a number of biological processes and in doing so, have aided in the discovery and cure of a multitude of diseases, thus saving lives (Wangler et al., 2017). Many different types of model organisms exist, each bringing a unique contribution to the field, the most common ones include: *Mus musculus*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, *Saccharomyces cerevisiae*, and *Danio rerio*. Clearly these are quite different from another, yet despite their obvious differences, they all share the following characteristics, albeit in varying degrees: easy to handle and care for, have a relatively short lifespan, rapidly reproduce, have conserved traits with humans, easy to genetically manipulate and often don't have the same ethical concerns as humans. Model organisms also allow researchers to study otherwise complex traits, cellular processes or molecular mechanisms in a simplified context. *C. elegans* for example share many different genes with humans but don't have as many controlling a given trait, this allows researchers to pinpoint the function of a gene without having to worry about other genes masking its effects. Even if we were to exclude the ethical concerns from humans, it simply wouldn't be practical to use them. It would take 9 months to study embryogenesis in humans but only 19 days in mice, and other long term phenomenons, such as cancer would also be rather impractical to study in humans (Murray et al., 2010 & de Jong et al., 2010). All of the above should be carefully considered when selecting a model organisms. Each model will be better suited for different types of research and there will never be a single one that fits everyone's needs.

Danio rerio, also known as zebrafish, have been quickly growing in popularity amongst the scientific community, and for good reason. They are an excellent model to study the process of solid tumor formation, metastasis and tissue regeneration (Teame et al., 2019). They are also quite easy to care for, reproduce and genetically manipulate. Despite their numerous benefits, zebrafish have a major drawback, the commonly used strains have a high degree of genetic heterozygosity. An underlying requirement of model organisms, and one that doesn't get much attention, is that of genetic homogeneity (Barre'-Sinoussi et al., 2015). In an ideal experiment, all of the individuals would be genetically identical to one another, this would ensure that any seen effect is due to the tested variable and not to biological differences. As such, genetic variation in a model is an incredibly important factor to keep in mind, and one that should be kept to a minimum when possible. Indeed, a high degree of genetic variation in the population of used individuals can confound one's results, leading to potential false-positives or false negatives (Liu et al., 2013). Because of this, it has become common practice to inbreed mice in order to minimize the amount of biological differences between individuals, thus limiting confounding variables (Lutz et al., 2012).

The lack of a zebrafish strain with a low degree of genetic heterozygosity has been a major shortcoming of the model. To circumvent this problem, Dr. Sakai and his lab have developed 2 highly homozygous strains of zebrafish via inbreeding. The commonly used zebrafish strains AB and India were inbred via full sibling pair mating to generate ABM and IM respectively. ABM has been inbred for 28 generations while IM has been inbred for 50 generations. Though these inbred zebrafish do indeed have a high degree of genetic homogeneity, their lifespan has been shortened by about one third. Interestingly, this decrease in lifespan is seemingly not due to any obvious health issues. This last point is quite entrancing and inconsistent with the literature (Kelly, 2000). Many other organisms have a shorter life as a product of inbreeding, yet they often die prematurely due to some health related disease. The inbreeding of dogs is a perfect example (Yordy et al., 2019). This however doesn't seem to be the case for ABM and IM. No

obvious health related issue makes them succumb to their death, but rather, they seem to age prematurely. This is supported by the observation that they develop many of the characteristics of aging at an accelerated rate, namely a hunched back and lighter coloration (Kishi et al., 2009). Several reasons could explain why these inbred Zebrafish have a shortened lifespan, that of shortened or aberrant telomeres is one of the most exciting to think about.

Telomeres are the physical ends of our chromosomes. They prevent our genome from being sensed as double strand breaks (DSBs) and in doing so, prevent illegitimate DNA damage repair from occurring (Shammas, 2012). This is incredibly important as DSB repair mechanisms directed towards telomeres may lead to chromosome-to-chromosome fusions and breakage-fusion-bridge cycles. Both have been implicated to have a causative role in cancer (Dhillon et al., 2016). Perhaps an equally important function of telomeres is that of acting as biological clocks (Oeseburg et al., 2010). Due to the end replication problem, telomeres progressively get shorter with each replication cycle until they get so short that the cell either undergoes apoptosis or becomes senescent (Sell, 2007). While this may seem like a bad thing, it's actually a major anticancer mechanism which prevents cells from living too long and ultimately being the demise of the organism (Hornsby, 2016). While the dynamics of this process are quite different from humans to zebrafish, telomeres are still the chief cellular timekeepers in both (Carneiro et al., 2016). The shortening of telomeres has been implicated with aging as well as a number of age related diseases in both humans and zebrafish (Anchelin et al., 2011). These two facts, along with the observation that the inbred strains don't prematurely die of any distinguishable cause but exhibit a seemingly accelerated aging phenotype, led to the hypothesis that the inbred strains of zebrafish prematurely age as a consequence of aberrant telomere maintenance mechanisms which culminated in short or aberrant telomeres. In order to find evidence in support of this claim, we intended to measure the telomere length of both AB and ABM 5 days post fertilization (5 dpf) and 8 months post fertilization (8 mpf) via quantitative polymerase chain reaction (qPCR) based on previously

described work (Cawthon, 2002). Collectively, this information was meant to give insights on whether the inbred strain had inherited short telomeres and whether the dynamics of telomere maintenance were altered in any way. In this report, we found no significant difference in telomere length between AB and ABM 5 days post fertilization and were unable to generate reliable data from the 8 mpf zebrafish.

Methods

Experimental design

In order to investigate whether the process of inbreeding led to inherently shorter telomeres, we decided to measure the telomere length of zebrafish 5 dpf. This time frame was thought to be suitable given that the larvae would be large enough to extract significant amounts of DNA but still young enough to have an accurate reflection of the telomere length at fertilization. This essentially tells us if ABM start their lives with shorter telomeres. Next, we intended to measure the telomere length of zebrafish 8 mpf, this would tell us if the dynamics of telomere length maintenance were aberrant in any way.

Animal housing

Danio rerio were housed in standard conditions at the National Institute of Genetics, Mishima, Japan. Adults were housed in circulatory systems at 28°C while larvae were housed in petri dishes and placed in an incubator to ensure a constant temperature of 28°C. Both followed a light:dark cycle of 12:12. Adults were fed a combination of live brine shrimp and generic fish food while larvae were not fed given that they were euthanized 5 dpf.

Breeding pairs and care of fertilized eggs

A single male and female were selected from the same communal housing and placed in a breeder tank anytime after 17:00. Males with a red tint, indicative of androgens, and females with an extended abdomen were selected as good candidates to yield fertilized eggs. The pair would normally spawn around 11:00 and the fertilized eggs were collected within 3 hours of spawning. The pairs were returned to their respective tanks and the fertilized and unfertilized eggs placed in a petri dish and stored in an incubator at 28°C. Approximately 6 hours post fertilization, the eggs were sorted and any infected, dying or unfertilized eggs were removed from the dish in order to prevent others from dying. 48 hours post fertilization the eggs would hatch, and chorion manually removed. Larvae were monitored daily until 5 dpf. The water was also changed every day until euthanization.

Euthanization of zebrafish 5 dpf and DNA extraction

Zebrafish 5 dpf were anesthetized using Ethyl P-Aminobenzoate at a concentration of 1:1000. After 5 minutes in anesthetic, some crushed ice was added to the dish and the dish was also placed on ice for an additional 10 minutes. Fish were confirmed to be euthanized by tapping on petri dishes and observing a lack of movement in addition to the fact that they would sink. The fish were then aspirated using a pipet and the water was strained by using a 80um nylon mesh. They were then transferred to a 2 ml tube and weighed. The DNA was extracted using the commercially available kit from QIAGEN: QIAamp DNA mini Kit, with some slight modifications. For every 25 mg, 20 ul of proteinase K and 180 ul of ATL buffer were added, the samples were then placed at 56°C with some light agitation. Tissue lysis took approximately 5 hours but samples may be left shaking at 56°C overnight as well. The remainder of the DNA extraction was performed as described by the protocol, the RNase step was performed in order to remove any RNA which may interfere with the qPCR. DNA concentration was determined using

QuantiFluor as described by the protocol. The quality of the DNA was also assessed via NanoDrop and the size of the eluted DNA was determined via agarose gel electrophoresis as previously described (Davis et al., 1986).

8 mpf fin DNA extraction

8 mpf AB and ABM were anesthetized in Ethyl P-Aminobenzoate at a concentration of 1:1000. This normally took a few minutes, fish would often turn right side up and breathe slowly. Prior to taking a fin clip, the fish were determined to be under anesthesia by pinching their tail fin, after which a section of their tail fin would be cut and stored in a 2 ml tube. Fish were immediately placed in a container with fresh water and monitored for a few hours before returning them to their respective tanks. 25 mg of tissue were rarely obtained from a single fish, nevertheless, the samples were weighed and 20 ul of proteinase K along with 180 ul of ATL buffer were added to the tube. The samples were then placed at 56°C shaking. Full lysis of tissue typically took about 2 hours but may be left overnight as well. The remainder of the DNA extraction process was performed as described by the protocol, the RNase step was performed in order to remove any RNA which may interfere with the qPCR. DNA concentration was determined using QuantiFluor as described by the protocol.

qPCR conditions

In order to measure the telomere length of AB and ABM, quantitative polymerase chain reaction (qPCR) was employed based on the original qPCR method for telomere length measurement (Cawthon, 2002). TB Green *Premix Ex Taq II* (Takara) and the Roche LightCycler 480 were used for all experiments using qPCR. Each sample was loaded in triplicates and had a total of 4 biological replicates. Given that both a different polymerase and thermal cycler were used, a variety of different conditions were tested before finding the best one, these are summarized in

figure 2. The qPCR that generated the most consistent results were the following: 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec, 56°C for 30 sec and 70°C for 10 sec.

The following primers were used based on previous work (Evans et al., 2021):

Telomere forward: GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT

Telomere reverse: TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA

C fos forward: CAGCTCCACCACACTGAAGA

C fos reverse: GCTCCAGGTCAGTGTTAGCC

Figures and statistical analysis

All figures and statistical analysis were carried out using Prism version 9. An unpaired, student's t test was used in order to determine significance.

Results

In order to ensure the DNA extraction method didn't shear the DNA sample, we performed agarose gel electrophoresis on our DNA extract, figure 1. The presence of a single band indicates the presence of a single product size. The product was estimated to have a molecular weight of 20kb based on the molecular weight ladder. Samples were run on a 0.5% agarose gel infused with ethidium bromide for 30 minutes at 230V.

After we confirmed our DNA extraction method to yield a single sized product, we ran our first qPCR reaction. A table summarizing all of the different conditions tried can be found in figure 2. Given that the original paper describing telomere length measurement using qPCR used a two step qPCR, an adaptation of this was used for both telomere and single copy gene, c fos, amplification. In order to determine the specificity of our qPCR reaction, melting curve analysis was employed for both telomere and c fos amplification figures 3 and 4. The 2 step qPCR telomere amplification yielded a shoulder, suggesting different products were being made, the

amplification for c fos yielded primer dimers, illustrated by the small peak at a lower temperature. Because of this, we opted to try the 1 minute 3 step qPCR which didn't have the mentioned problems. We also decided to confirm the size of the qPCR amplicons via agarose gel electrophoresis, figure 5. Amplicons were expected to have a size of 67kb for telomere products and 175 kb for c fos products. As expected, the gel for telomere products showed a smear with a faint band localizing at 67 kb. Since the 3 step qPCR didn't seem to have as many problems as the 2 step did, we decided to proceed with the 3 step qPCR and did a standard curve analysis using different concentrations of DNA. To our surprise, the efficiencies did not fall into acceptable ranges, suggesting that there was still something wrong with our set up. To this end, we tried to decrease the extension times in order to obtain good efficiencies. An interesting trend arose; as we decreased the extension time, the efficiencies got better and the melting curves show a smaller product being made, figure 6. Figure 7 illustrates the standard curves derived from the different elongation times, much like the melting curves, the efficiencies of the qPCR reaction improve as the extension time is lowered. Given that the smallest product size is to be expected, (Cawthon, 2002), we used the 10 second extension time conditions to analyze the telomere length of AB and ABM. The 1 minute extension time is ideal for c fos amplification and was kept as such for all qPCR reactions, a representative image is found in figure 8. A summary of all the qPCR efficiencies is found in figure 9, qPCR reactions with efficiencies falling between 3.0-3.6 were deemed to be acceptable, those highlighted in red are not acceptable. Though a variety of different formulas could be used to quantify the obtained data. The ΔCt , and $1/(\Delta Ct \text{ telomere} / \Delta Ct \text{ C fos})$ were used based on previous publications, figure 10, (Cawthon, 2002 and Evans et al., 2021). In either case, we were unable to detect a statistically significant difference between the AB and ABM 5 dpf. Due to the poor efficiencies of the 8 mpf samples, we opted not to analyze the data as any interpretation would be meaningless, the poor efficiency is indicative of a problem with the amplification; as such the data is likely not an accurate reflection of the relative telomere length.

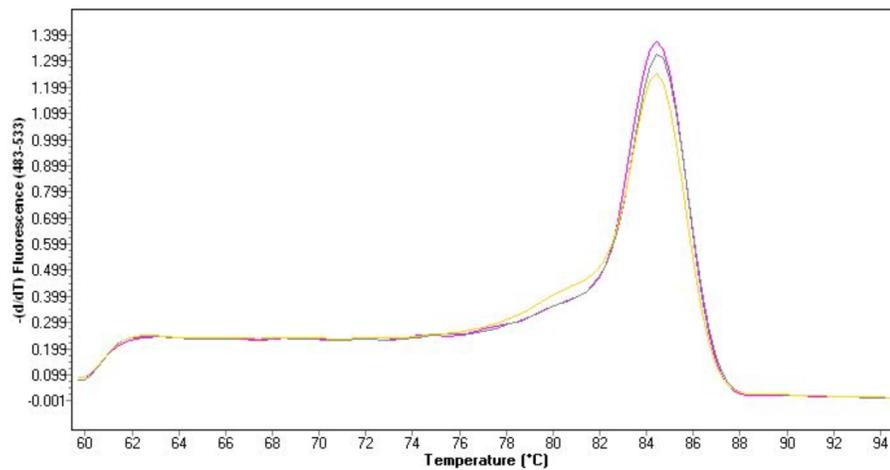


Figure 1. 0.5% agarose gel infused with ethidium bromide. Lane 1 was loaded with a molecular weight ladder, highest band containing a product of 15kb. Lane 2 was loaded with DNA extracted from AB 5 dpf, lane 3 was loaded with DNA extracted from ABM 5 dpf.

conditions	Activation step	# of Cycles	denaturing	annealing	elongation
Per protocol	95°C , 30 s	40 cycles	95°C 10s	54°C 1 min	
1 min elongation	95°C , 30 s	40 cycles	95°C 10s	54°C 30 s	72°C 1 min
30 sec elongation	95°C , 30 s	40 cycles	95°C 10s	54°C 30 s	72°C 30 sec
15 sec elongation	95°C , 30 s	40 cycles	95°C 10s	54°C 30 s	72°C 15 sec
10 sec elongation	95°C , 30 s	40 cycles	95°C 10s	54°C 30 s	72°C 10 sec

Figure 2. Summary of different qPCR conditions tried. The 10 second elongation conditions yielded the most consistent results.

3A



3B

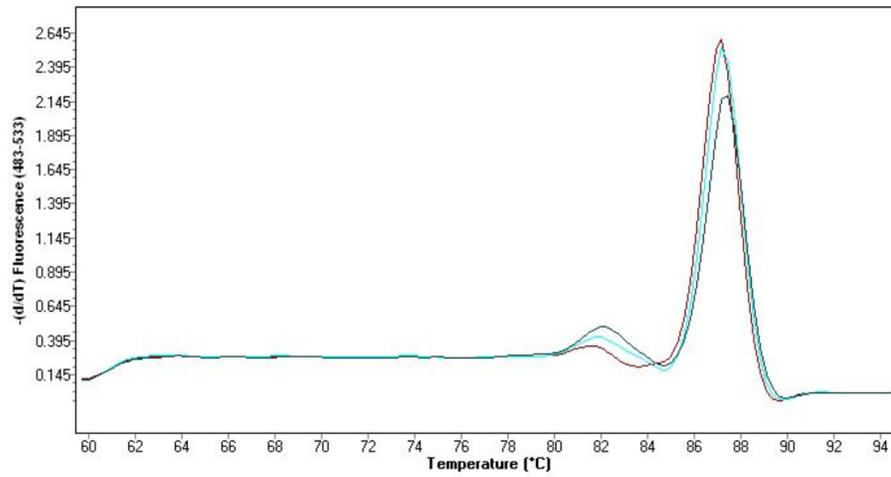
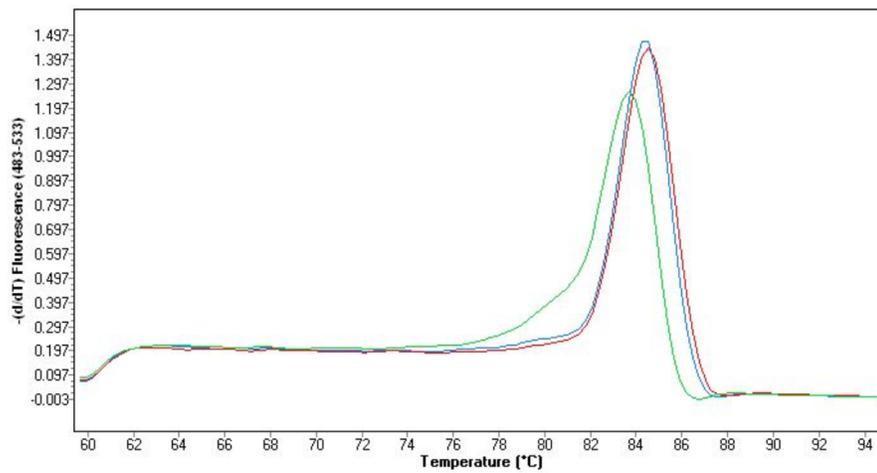


Figure 3. 2 step qPCR melting curves. **3A** melting curve analysis for telomere amplification. **3B** melting curve analysis for c fos amplification.

4A



4B

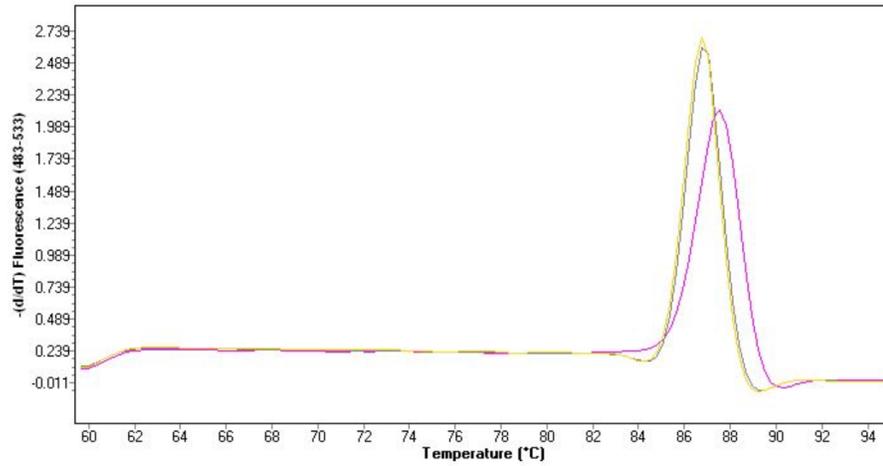
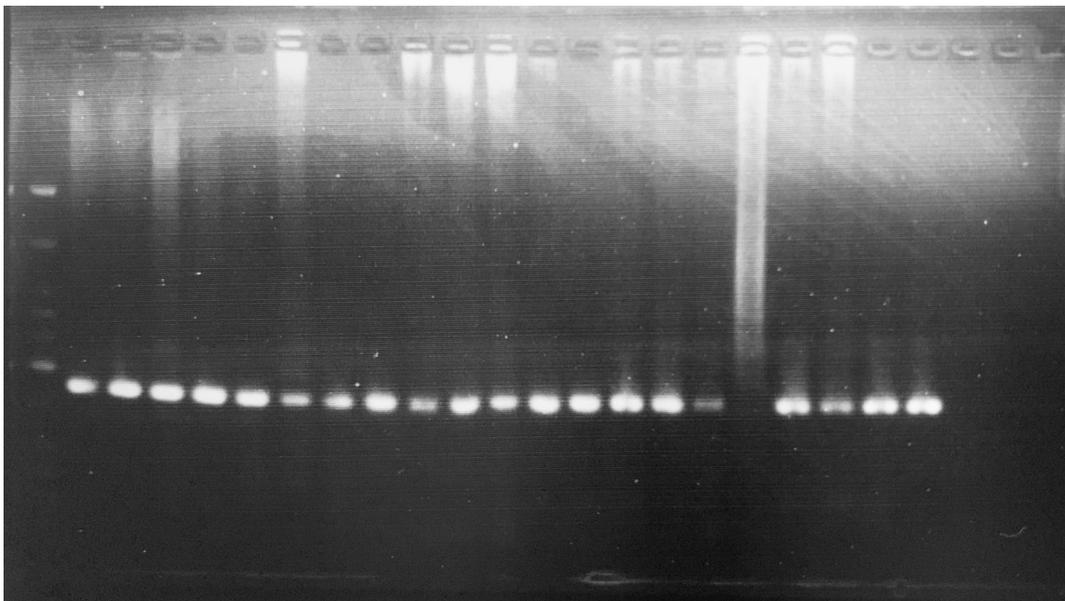


Figure 4. 3 step qPCR melting curves. **4A** melting curve analysis for telomere amplification. **4B** melting curve analysis for c fos amplification.

5A



5B

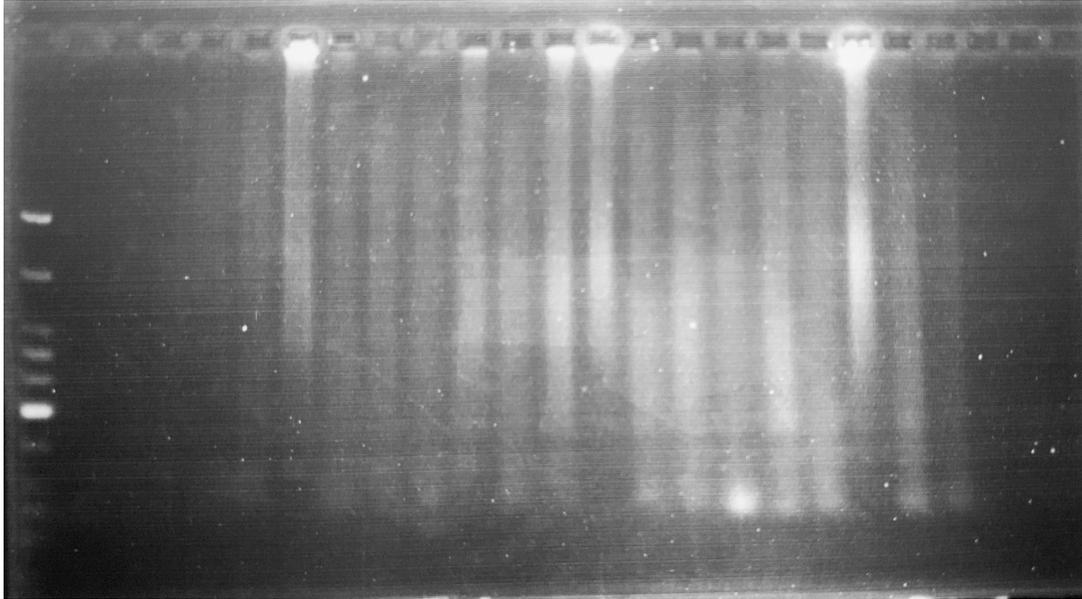
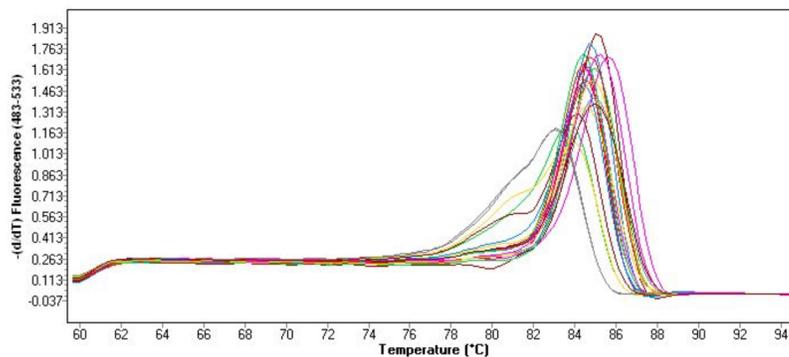
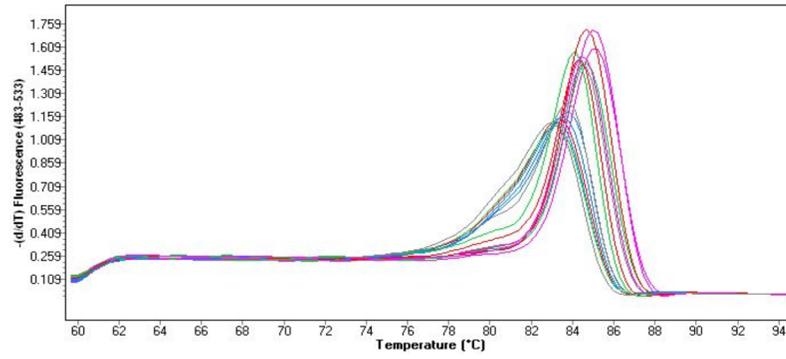


Figure 5. 3% agarose gel electrophoresis for qPCR products. **5A** products of *c fos* amplification, single crisp band near 167bp, as expected. **5B** products of telomere amplification produce a smear with a feint band near 67 bp, as expected.

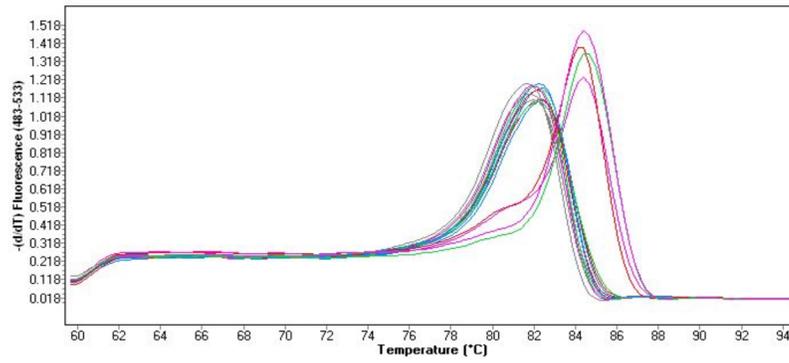
6A.



6B.



6C.



6D.

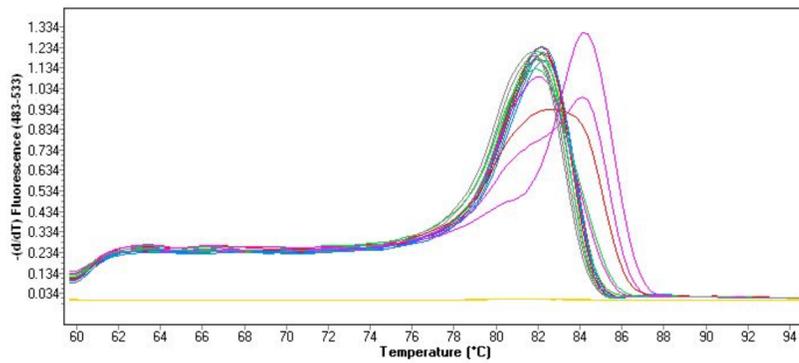
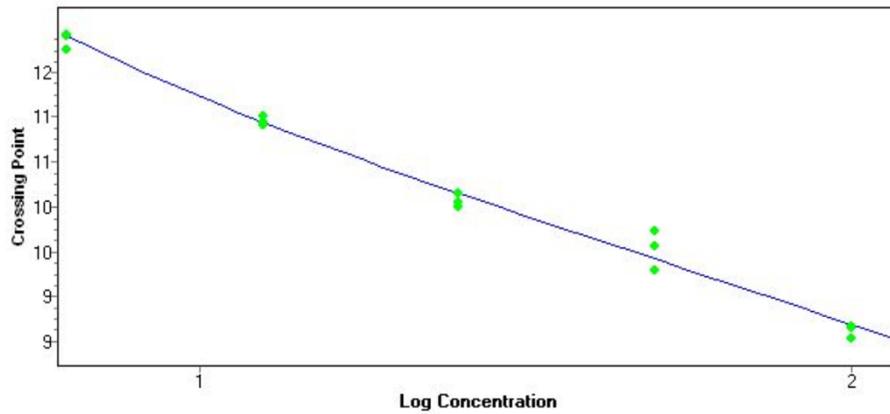


Figure 6. Melting curve analysis for the various products in the different qPCR conditions.

6A: 1 minute elongation shows larger products predominantly being made. **6B:** 30 second elongation shows a mix of larger and smaller products being made. **6C:** 15 second elongation shows the smaller products predominantly being made. **6D:** 10 second elongation shows the smaller products almost exclusively being made.

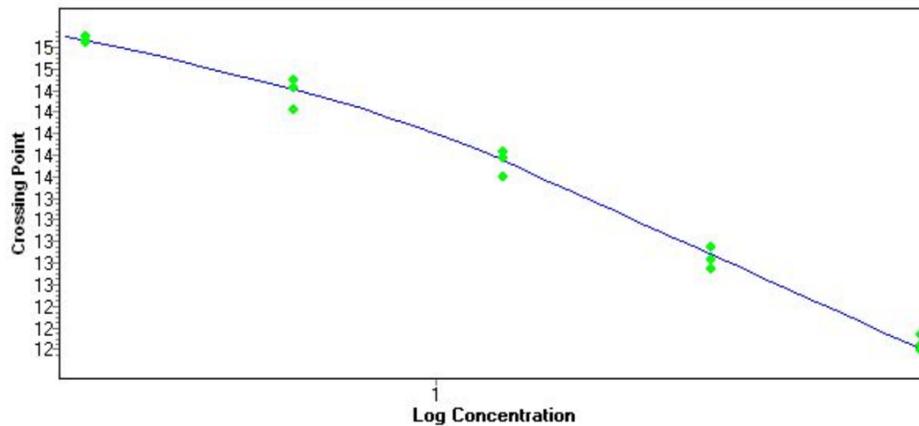
7A

Error: 0.0147
Efficiency: 2.600
Slope: -2.409
YIntercept: 13.51
Link: 27.31



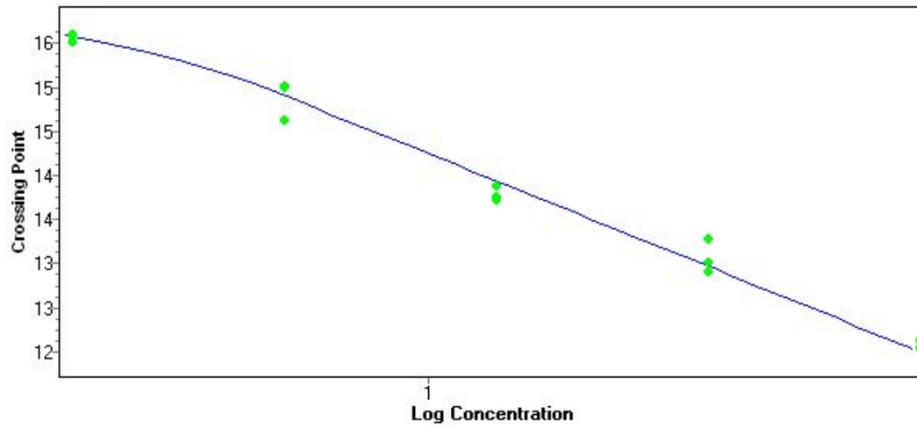
7B

Error: 0.00464
Efficiency: 2.213
Slope: -2.898
YIntercept: 16.93
Link: 12.26



7C

Error: 0.0224
Efficiency: 2.045
Slope: -3.219
YIntercept: 17.47
Link: 5.871



7D.

Error: 0.0104
Efficiency: 2.009
Slope: -3.301
YIntercept: 17.41
Link: 0.000

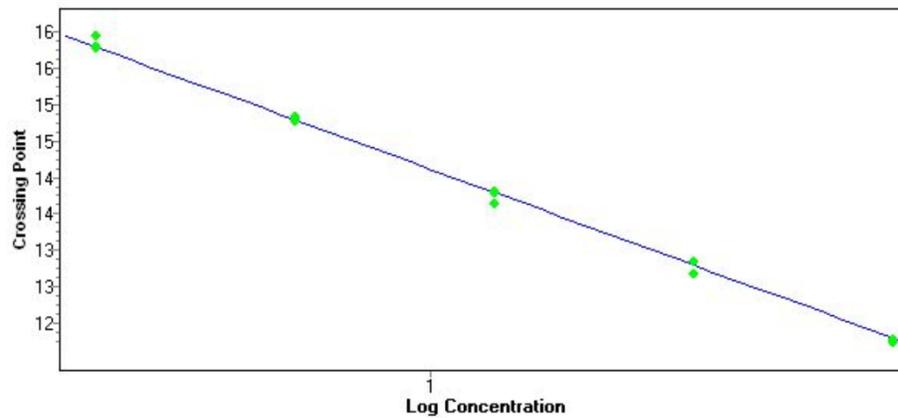


Figure 7. Standard curve analysis using DNA concentrations ranging from 50, 25, 12.5, 6.25 and 3.125 under different qPCR conditions. As the extension time is decreased, the efficiency approaches 2.000, indicative ideal conditions **7A.** 1 minute elongation time. **7B.** 30 second elongation time. **7C.** 15 second elongation time **7D.** 10 second elongation time.

Error: 0.00153
 Efficiency: 1.969
 Slope: -3.399
 Yintercept: 26.27
 Link: 25.00

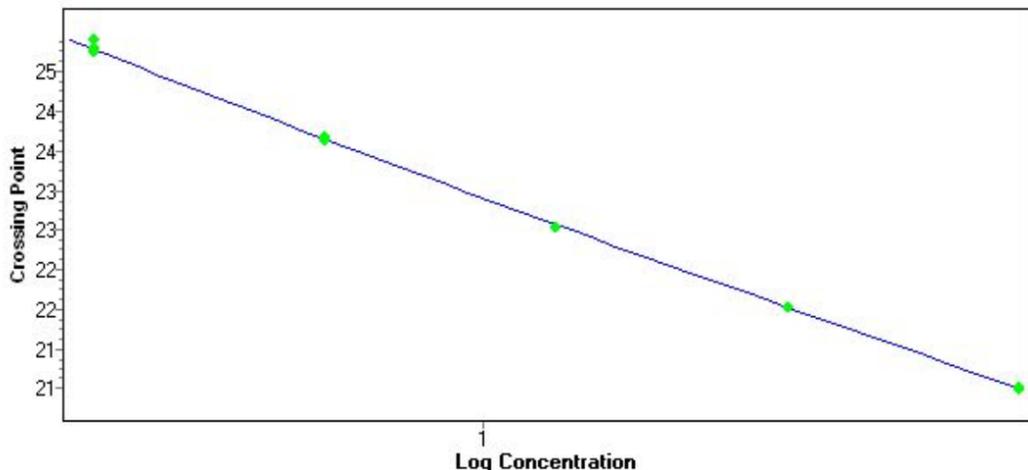


Figure 8. Representative standard curve analysis for c fos. The 1 minute elongation time was ideal for c fos amplification and rarely yielded poor efficiencies.

9A

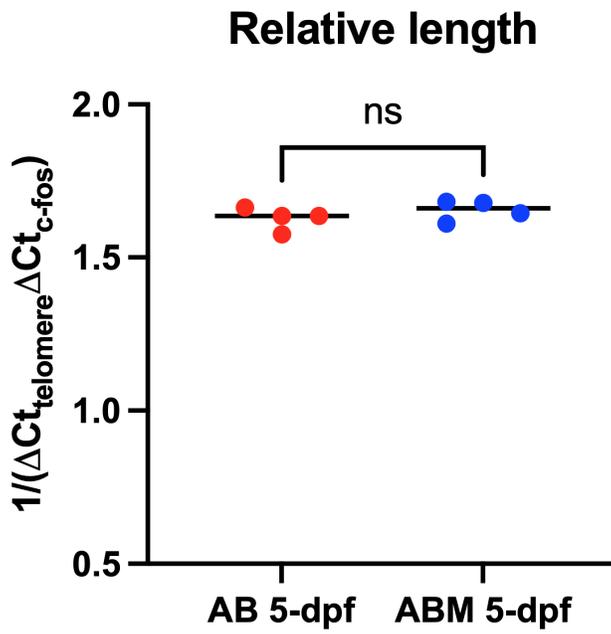
5 dpf	Efficiency telomere	Slope telomere	Efficiency C fos	Slope C fos
AB 1	1.969	-3.399	1.896	-3.6
AB 2	2.044	-3.22	1.889	-3.621
AB 3	2.156	-2.997	1.902	-3.582
AB 4	2.064	-3.177	1.902	-3.58
5 dpf	Efficiency telomere	Slope telomere	Efficiency C fos	Slope C fos
ABM 1	2.009	-3.301	1.806	-3.896
ABM 2	2.137	-3.032	1.969	-3.399
ABM 3	2.362	-2.678	1.914	-3.548
ABM 4	2.206	-2.911	1.922	-3.523

9B

8 mpf	Efficiency telomere	Slope telomere	Efficiency C fos	Slope C fos
AB 1	2.448	-2.572	1.982	-3.365
AB 2	2.191	-2.936	1.95	-3.447
AB 3	2.239	-2.856	1.953	-3.439
AB 4	2.049	-3.21	2.008	-3.302
8 mpf	Efficiency telomere	Slope telomere	Efficiency C fos	Slope C fos
ABM 1	2.448	-2.572	1.999	-3.324
ABM 2	2.886	-2.173	2	-3.322
ABM 3	2.726	-2.296	1.933	-3.493
ABM 4	2.422	-2.603	1.977	-3.378

Figure 9. Tables summarizing the efficiencies and slopes of the standard curves for AB and ABM, both 5 dpf and 8 mpf. **9A:** 4 biological replicates for AB and ABM 5 dpf. **9B.** 4 biological replicates for AB and ABM mpf, 2 males and 2 females each. Numbers highlighted in red indicate unacceptable efficiencies.

10A



10B

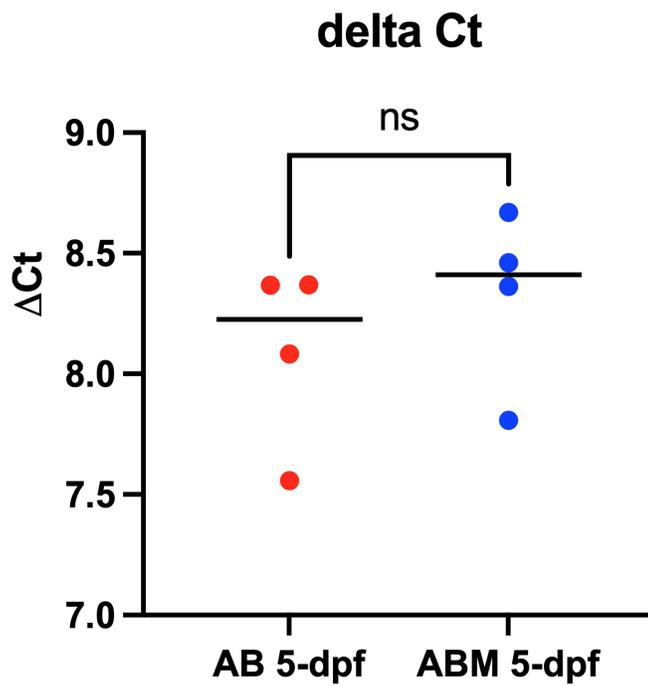


Figure 10. Quantification of telomere length via: **10A:** $1/(\Delta \text{ct telomere}/\Delta \text{ct c fos})$ and **10B:** Δct . Regardless of method employed, no detectable difference in telomere length was observed between AB and ABM at 5 dpf.

Discussion & Conclusions

Despite not finding any differences in the telomere length of ABM compared to AB, the hypothesis that shortened telomeres have a role in the prematurely aging phenotype of ABM cannot be completely excluded. Having only been able to measure the telomere length 5 dpf, it is still possible that the inbred strain inherited aberrant telomere maintenance genes which result in the inability to maintain properly functioning telomeres and telomere length later in life. This hypothesis was meant to be tested by measuring the telomere length 8 mpf. As already mentioned, we were unable to reliably measure the telomere length 8 mpf for unknown reasons. All of the DNA samples were extracted using the same kit and there were no differences in the quality of the DNA. The idea that differences in DNA quality are not responsible for the inability to generate proper standard curves is supported by the fact that c fos is properly amplified in both 5 dpf and 8 mpf samples. As such, it's possible that some differences in the telomeres of zebrafish 8 mpf are responsible for the discrepancy. This is quite troublesome, if true, perhaps using different qPCR conditions for the zebrafish 8 mpf could mitigate this problem. Interestingly, when looking at the qPCR efficiencies for both 5 dpf and 8 mpf, it seems like the amplification efficiencies for c fos are closer to ideal in the 8 mpf despite not having acceptable telomere amplification efficiencies; conversely, the amplification efficiencies for c fos are further from ideal in the 5 dpf samples despite having acceptable telomere amplification efficiencies. Even more troublesome, and yet another clue suggesting intrinsic telomeric differences. qPCR for telomere length measurement is a quite difficult experiment to set up; while it is simpler than other

methods, numerous factors likely play a role in the inability to generate reliable and reproducible results in the zebrafish 8 mpf. For starters, the repetitive nature of telomeres allows the primers to bind essentially anywhere along the sequence, thus producing multiple different products as seen by the smear in figure 5B. This is one of the reasons why people thought it impossible to measure telomere length via qPCR prior to Cawthon, 2002. Reducing the elongation time reduces the likelihood of amplifying larger amplicons given that the polymerase would not have enough time to amplify such large stretches of telomeric sequences, because of this, smaller amplicons are predominantly formed, as seen in figure 6. Even with the shorter elongation time, larger fragments can be made due to some stochastic events, though it is less likely. If the polymerase is able to amplify a larger sequence in the earlier cycles, then all the subsequent cycles will also produce larger amplicons since the starting template would be larger. This alone introduces a great deal of variation in one's assay, and when trying to detect even the smallest of differences in telomere length, the less variation the better. Other methods to measure telomere length should be explored, terminal restriction fragment, TRF, followed by southern blotting is one of the most accurate ways to measure telomere length and unlike the qPCR method, yields absolute telomere length, not relative (Kimura et al., 2010).

Another thing worth mentioning is the fact that while disease stemming from aberrant or short telomeres exist, short telomeres per se aren't the cause behind the disease, but rather, the consequences that come as a result of short telomeres, namely senescence (McHugh et al., 2018).

Senescence is a state of permanent proliferative arrest that cells encounter in response to a number of factors such as: stress, oncogene activation, extensive DNA damage and short telomeres (Di Micco et al., 2022). This permanent proliferative arrest is followed by a change in metabolic activity where the cells cease to partake in their canonical function and start exhibiting a metabolic phenotype known as the Senescence Associated Secretory Phenotype or SASP, this has been shown to have detrimental effects on organismal health and lead to aging (Kumari

et al., 2021). Even if we did find that the ABM strain had shorter telomeres compared to AB, this alone wouldn't explain why these zebrafish supposedly premature age, it would however provide us with a strong reason to believe the ABM strain encounters a greater degree of replicative senescence, which would explain why these zebrafish live less.

As already mentioned, various different types of senescence exist and should be considered as potential reasons why ABM prematurely age, senescence can be detected via the presence of beta-galactosidase regardless of the type of senescence (Debacq-Chainiaux, et al., 2009).

We should also consider the possibility that other factors are responsible for the prematurely aging phenotype of these zebrafish. The simplest one being that of them dying from some health related disease. While the ABM zebrafish do exhibit many of the hallmarks of aging at an accelerated rate, the lack of an assay that can identify that cause of their death prevents us from excluding the possibility of them dying from a heart attack for example rather than being a prematurely aging model. Future directions should include ways to exclude this from being a possibility.

Lastly, loss of heterogeneity has been shown to be detrimental for the health of organisms (H. Schwarzenbach, 2013). Humans for example are heterozygous for numerous genes which would be detrimental if homozygous, careful consideration should be put into the possibility that ABM prematurely age due to a loss in heterogeneity of particular genes.

In conclusion, we detected no significant differences in the telomere length of ABM compared to AB at 5 dpf and were unable to measure their telomere lengths at 8 mpf, likely because of some intrinsic differences in the telomeres between AB and ABM which require different qPCR conditions.

Acknowledgements

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