Early life stress and telomere length in cichlids

by

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Abstract

Early life exposure to stress can have life-long effects on an organism. These effects may be detrimental, or they may be adaptive if they allow the organism to develop an adult phenotype which better matches the environment predicted by the stressor. Early life stress influences many traits, including growth rate and stress coping style. Early life is suggested as a time where oxidative stress increases due to the increased metabolic growth demand. Higher growth rates impose increased oxidative stress, which has been linked to telomere shortening. Here I examine the link between age, growth, stress, coping style, and telomere length. In the first study I quantified telomere length in cichlid species *Astatotilapia burtoni* across four different age groups. In the second study I quantified behaviour, and cortisol hormone levels and telomere per single gene copy in two types of convict cichlid (*Amatitlania nigrofasciata)* tissue for stress treated and control fish. I found that liver telomere length was not related to age in *A. burtoni* but fin telomere length did increase with age in fin tissue. In *A. nigrofasciata* we found that there was no relationship between stress treatment, growth rate or residual body condition. There was no significant difference between the stress treatment and control groups relative telomere length in fin or liver tissue. Principal components of the behavioural measures showed that there was an interaction of stress treatment, fin telomere length and PCA components. Fish in the stress treatment group with longer fin telomeres tended to be less exploratory and have a more reactive coping style than controls. These results suggest that telomeres in cichlids do not follow the same pattern of attrition as found in other species such as birds and humans. The different pattern of telomere dynamics found in cichlids could be suggestive of high levels of telomerase expression in somatic tissue due to their ability to grow indeterminately.

Preface

This thesis is an original work by Kennedy Fjellner. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Early-life stress and Telomere length”, AUP00000055, 11/24/2017.

This thesis is an original work by Kennedy Fjellner. No part of this thesis has been previously published.

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List of Abbreviations

Cq – Fractional cycle number
HPA – The hypothalamic- pituitary- adrenal axis
HPI – The hypothalamic–pituitary–interrenal axis
HPLC – High-performance liquid chromatography
qPCR- quantitative or real time polymerase chain reaction
PCR – polymerase chain reaction
pUC - "p" prefix (denoting "plasmid") and the abbreviation for the University of California
qPCR- quantitative or real time polymerase chain reaction
ROS – Reactive Oxygen Species
SCG – A single copy gene
TA – Telomerase Activity
TRF – Terminal restriction fragmentation
TMS – Tricaine mesylate (Tricaine methanesulfonate, TMS, MS-222)
T/S ratio- Telomere repeats (T) per number of reference gene copies (S)

Chapter 1: General Introduction

General Introduction

Stress is often used as a catch-all term to encompass a wide range of behavioural and physiological effects. Stress exposure, specifically in early life, can influence a wide range of traits that may impact the entire life course. Long-term effects of stress exposure in early pre and post-natal life can increase glucocorticoid hormones and influence stress responsiveness in adulthood (Monaghan & Haussmann, 2015). Hormones involved in the stress pathways play an important role in homeostatic energy management, and have been proposed to be the mechanism by which the offspring is informed about the postnatal environment (Sheriff & Love, 2013; Marshall & Uller, 2007). Individuals who are exposed to stress early in life may have phenotypic changes to their behaviour and stress coping style. Coping style being the animal's ability to use specific behaviours or strategies to adapt to and overcome stressful encounters. These changes can be adaptive if the environmental context matches what they were exposed to but maladaptive if the contexts do not match. For example, if an animal does not experience any stress in early life but the environmental context is harsh and unpredictable, they will struggle to deal with this environment. However, if the early life of an organism is stressful and the environment they experience as an adult is not stressful, they may experience a heightened or exaggerated stress response unnecessarily that may resemble an anxiety-like response (Nederhof & Schmidt, 2012). Stressful conditions early in life can give rise to exaggerated stress responses that do not match the situation at hand and have been experimentally shown in zebra finches to adversely affect health and longevity (Monaghan et al. 2012).

Experiences in early life confer the ability for animals to cope with environmental stressors by informing them about the environment. The ability to cope with stress in the

environment significantly impacts health and fitness. Stress refers to normal reactions to the environment that are generally adaptive, whereas distress generally occurs when the threat is very severe or long term (National Research Council (US) Committee on Recognition and Alleviation of Distress in Laboratory Animals, 2008*)*. An animal's coping style is a consistent set of behavioural and neuroendocrine responses that allow it to deal with the environment (Koolhaas, 1999). Coping often depends on the controllability and predictability of the stressor. Coping style in animals is generally divided into two main behavioural and physiological stress response patterns: Proactive coping and reactive coping (Koolhaas, 1999). Proactive coping styles include confronting stressors more frequently, lower latency to aggression, and lower latency to explore a novel environment (Øverli et al., 2007; Sih et al., 2003; Sih et al., 2004; Koolhaas, 1999). Whereas reactive coping styles have a longer latency to aggression, and increased freezing/hiding (Øverli et al., 2007; Koolhaas, 1999). High HPA or HPI activation is seen with more reactive coping/high-anxiety and low activation associated with proactive/low-anxiety behaviours (Øverli et al., 2007; Sih et al., 2003; Sih et al., 2004; Koolhaas, 1999). We can operationalize these behaviours using assays like the open field and plus maze task. These tasks measure locomotor behaviour and exploration which is the pattern of responses to novel situations. Exploration in animals generally encompasses behaviours like freezing/hiding, scototaxis – preference for dark/light areas; Maximino et al., 2010a; Maximino et al., 2010b) – and thigmotaxis – travelling close to walls (Réale et al., 2007; Champagne, Hoefnagels, de Kloet, & Richardson, 2010).These operationalized behaviours generally fall into proactive coping with higher locomotor activity and lower latency to explore novel environments and reactive coping with increased scototaxis, thigmotaxis, lower locomotor activity and slower latency to explore novel environments.

Proactive and reactive copings styles have also been linked to the bold-shy continuum a well-studied personality dimension in fish (Toms, Echevarria, & Jouandot, 2010). The bold-shy continuum commonly measures boldness in fish with assays such as latency to approach a novel object, explore a novel environment, or emerge from a shelter (Toms et al., 2010). The bold-shy continuum is also closely correlated with both coping style and the pace-of-life syndrome hypothesis (POLS). POLS predicts that shyness and boldness covary on a slow-fast continuum. Bolder individuals expend more energy on locomotor activity and aggression, using more energy than shyer individuals. POLS was originally developed to understand life history trade-offs strategies at the population level however it is now used to look at within population strategies for resource allocation (Mathot & Frankenhuis, 2018). Increasing evidence suggests that shy-bold personalities are closely linked with growth rate, fecundity, and metabolism (Biro & Stamps, 2008)

Often animals' first experience with a stress is a high-risk environment like an encounter with a predator (Brown et al. 2014). Encounters with stress can be beneficial by gaining anti-predator skills or can be detrimental and result in neophobia which may impact foraging ability (Elvidge, Chuard, Pierre & Brown, 2016). Selye (1951) defined stress as "the non-specific response of the body to any demand placed upon it." This idea was refined to differentiate between "stressor" and "stress response". A stressor being a stimulus that threatens homeostasis and the stress response being how the organism reacts to regain homeostasis. Homeostasis is regulated by a complex system of behavioral and physiological systems within an organism to maintain optimal functioning (Chrousos, 2009; Romero, Dickens, & Cyr, 2009). Terms "distress" and "eustress" were used by Selye (1976) to distinguish between maladaptive and adaptive, aspects of stress respectively (Koolhaas et al. 2011). A more recent definition of stress

is a state in which homeostasis is either threatened or perceived to be threatened (Chrousos, 2009).

The physiological response to stress is a complex interaction of the endocrine and central nervous system. This response to stress is composed of interactions and feedback between the hypothalamus, pituitary and adrenal glands that can create hormonal cascades referred to as the hypothalamic-pituitary-adrenal (HPA) axis in mammals and the Hypothalamic-Pituitary-Interrenal Axis (HPI) in fish. The HPA or HPI axis is activated when a stressor is perceived, activating a flight or fight response, that reduce resources going to activities not necessary for survival. These unnecessary activities would include, but not be limited to growth, reproduction and body maintenance (Monaghan & Haussmann, 2015, Wingfield et al., 1998).

Hormesis is the concept that low doses of exposure to harmful substances can provide benefits while larger doses can cause harm. In the hormesis framework of stress low doses of exposure to stress can provide protective and beneficial responses where higher doses can be damaging to an individual or organism (Berry & López-Martínez, 2020; Sapolsky, 2000). Fat head minnows who were exposed to long term perceived predation stress returned to normal shoaling patterns quicker than controls (Meuthen et al., 2019). The hormesis theory suggests that there is an optimal level of stress that confers benefits throughout life and impact future behaviour. In zebra finches it was found that repeated mild stress exposure had beneficial lifespan effects (Marasco et al., 2015). Fish have also been seen to have enhanced reproductive performance when exposed to mild stress during maturation (Schreck, 2000; Schreck 2010). Time scale is also important when considering the dose-response of stress. Short term or acute exposure can confer some benefits (i.e. resiliency) while more chronic exposure can lead to damage. Russian sturgeon (*Acipenser gueldenstaedtii*) exposed to chronic levels of stress

associated with hatchery procedures such as high tank density, and high temperatures showed evidence of gamete quality deterioration (Bayunova, Barannikova, & Semenkova, 2002). However, dose-response may vary across taxa as some species may have different tolerances to stress (Schreck, Contreras-Sanchez & Fitzpatrick, 2001). Timing, level and maturational stage at which stress is experienced all contribute to the overall coping of the organism and its impact on future fitness.

Stress during development has been seen in previous studies to play an important role in future behaviour. When stress is experienced in early life behaviour can be altered (Clinchy, Sheriff and Zanette, 2013; Monaghan et al. 2012; Monaghan & Haussmann, 2015). Cichlids that were exposed to predator alarm cues as newly hatched wrigglers and juveniles showed significant avoidant responses, but adults did not (Joyce et al., 2016). Changes in behaviour show a developmental plasticity – changes in neural connections as a result of interactions with the environment – that act as a way to calibrate behaviour to match the local environment and create an adaptive phenotype (Carlson, 2017; Nederhof & Schmidt, 2012). With these behavioural changes we often see changes in HPA reactivity and higher levels of cortisol (Monaghan et al., 2012). Prenatal exposure to stress hormones can also have negative effects for threespined sticklebacks (*Gasterosteus aculeatus*), whose offspring did not show adaptive responses when they were exposed to a predator (McGhee, Pintor, Suhr, & Bell, 2012). There is also increasing evidence of transgenerational transmission of non-genetic effects that influence the phenotypic expression of offspring in covarying traits like growth, personality and fecundity (McCormick, 2009). Female threespined sticklebacks exposed to predation risk had offspring with higher concentrations of cortisol and as juveniles showed increased shoaling behaviour (Giesing, Suski, Warner, & Bell, 2010). Exposure to predator stress early in life has also been shown to influence

vulnerability to long term behaviour disruptions, glucocorticoid levels and changes in gene expression (Clinchy et al., 2011). Taken together it is possible that more physiological changes are taking places in addition to behavioural changes as a result of stress hormone exposure.

Telomere length is often used as a biomarker of health and fitness in an organism because it has been linked with lifespan and health span in humans and some vertebrates. Telomeres are the repetitive DNA that cap chromosomes and protect them from DNA loss during cellular replication. Telomeres are located at the end of eukaryotic chromosomes and are tandem TTAGGG repeats and their complements. As cells age, they can accumulate DNA damage that prevents them from functioning properly. In human stem cells the enzyme telomerase is responsible for elongating telomeres so that the cell can continue to replicate. Overtime cell turnover is limited by genetically determined and regulated mechanisms. These mechanisms control the telomere-telomerase system, which in turn controls the cells duplication capacity and its overall functionality (Mancini, 2009).

Alexey Olovnikov first proposed the concept of the 'mitotic clock' in 1971 suggesting that telomeres can limit the number of times a cell can replicate (Olovnikov, 1996). Each time a cell replicates the telomere loses a small portion of its genetic sequence, preventing any loss of the coding DNA. Part of the loss of the telomeric sequence occurs because the process of DNA replication is incomplete this is called the "end replication problem" (Levy et al. 1992). DNA molecules are made up of two strands of nucleotide sequences that run anti parallel to one another and during replication these two strands 'unzip" and each side is used as a template to make more DNA. The top strand (3' – 5') is the leading strand and is replicated continuously whereas the bottom strand (5'-3') end is replicated in small discontinuous steps. The enzyme attaches leaving that small section un-replicated.

Telomeres in human cells can shorten each replication cycle by about 30 – 200 base pairs (bp) per cell division but only about 10 of these base pairs are thought to be lost because of this end replication problem. However, telomeres can shorten much faster than could be explained by the incomplete replicative ability of DNA polymerase and the end replication problem. Mouse telomeres can shorten at a rate of 50-150 bases per cell division (Smogorzewska & de Lange 2004). This shows that telomere degradation must therefore be multifaceted and affected by additional factors. Degradation that is not accounted for by the end replication problem is thought to be due to or increased by reactive oxygen species (ROS) or free radicals (von Zglinicki, 2002). Telomeres can become mutated or broken when reactive oxygen species – a by-product of metabolism – build up in the cell. The enzyme telomerase builds back up telomeres lost due to degradation by ROS or replication. Telomerase is found in many tissues but in somatic cells it is found in low quantities. This is why somatic cells face telomere loss as cells divide. In stem cells, telomerases prevent telomeres from becoming too short while in somatic cells they simply cease replicating (Campisi, & di Fagagna, 2007).

Species lifespan usually varies by size with larger animals generally living longer than smaller ones (Calder, 1984). Larger animals, such as humans, generally have an increased temperature and metabolism because we are endotherms. Ectotherms, such as fish, have a generally lower body temperature that fluctuates with the environment (Johnston, & Dunn, 1987). Higher body temperature is also indicative of increased metabolism and therefore increased ROS (Gomes, Shay, & Wright, 2010). ROS are the result of unpaired electrons on oxygen molecules from cellular respiration. These ROS are highly unstable, they pass instability along by taking other electrons and triggering a damage cascade (Monaghan, Metcalfe & Torres 2009). This damaging cascade leads to increased oxidative stress. Oxidative stress is an

imbalance between oxidants (free radicals) and antioxidants, leading to an excess of oxidants that disrupts oxidation-reduction reactions in signalling proteins and can increase molecular damage (Sies, 2015). Early life has been suggested as a time during the life course where increased metabolic activity ramps up because of growth and increases ROS production (Monaghan, Metcalfe & Torres 2009).

Psychological stress may also shorten telomeres in humans by increasing oxidative stress caused by the release of cortisol (Epel et al., 2004). Glucocorticoids such as cortisol provide the body with glucose by tapping into protein stores via gluconeogenesis in the liver. This energy allows an individual fight or flee from a potential predator. Individuals that have been exposed to psychological stress have been shown to have higher levels of oxidative damage (Irie et al., 2003, Epel et al., 2004, Forlenza and Miller, 2006, Gidron et al., 2006). High levels of oxidative stress leading to telomere length attrition has been seen in animals as well. In king penguin chicks (*Aptenodytes patagonicus*) smaller chicks that grew faster showed higher oxidative damage and faster telomere length attrition (Geiger, et al., 2012). Wild blue tits (Cyanistes caeruleus) who were supplemented with antioxidants following a parasite treatment were found to have slowed telomere attrition (Badás et al., 2015). Supplementation with antioxidants has also be shown to reduce telomere loss in a range of taxa (Reviewed by Reichert, & Stier, 2017). Social stress or environmental stressors are generally what is thought of when we think of stress having negative effects, however, internal increases in metabolism or investment in other areas may also cause damage. Higher levels of oxidative stress have been found in brooding female *A. burtoni* compared to non-brooding females suggesting that there is a significant investment in reproduction (Sawecki, Miros, Border, & Dijkstra, 2019). The white-browed sparrow weaver (*Plocepasser mahali*) also shows that after breeding, females had increased oxidative damage

independent of their dominance status, but dominant females showed a marked reduction in plasma antioxidant defence over the course of the breeding season (Cram, Blount, & Young, 2015). Cichlids like *A. burtoni* and *A. nigrofaciata* have dynamic social hierarchies that determine their access to resources. Much like what has been seen in baboons (*Papio anubis*), maintaining a higher dominance level can have negative physiological implications from consistently defending their position (Sapolsky, 2005). In *A. Burtoni,* males can change reversibly status depending on the social landscape at the time, dominant fish regardless of treatment have been shown to have increased levels of reactive oxygen metabolites when compared to subordinate fish (Border, et al., 2019). Stress from defending territory, investing in growth or reproduction may increase oxidative damage or may be maintained but take resources away from antioxidant defence leading to delayed damage and telomere length attrition.

My primary objective in this thesis is to examine how telomere length varies among cichlids as a function of age, tissue and stress history. In chapter two, I first examined how telomere length varies at four different age groups to gauge how telomere length changes in non-stressed animals. I took terminal samples from different age groups of *A. burtoni* in order to determine how telomere length changes across the lifespan. This gives us a cross sectional picture of telomere length and rate of change across the lifespan. In chapter three I investigated how moderate early life stress changes telomere dynamics and growth in cichlids. The objective in this study is to determine if early-life stress has an impact on telomere length and how this stress will alter growth patterns. In this study I took a group of fry and split them into a control and treatment group. I then tested the fry on behavioural measures, took hormone samples and fin clips before the treatment group exposed to a net chasing procedure. After the treatment the

same procedure was done again to assess change between sampling. I conclude chapter four by discussing how these results relate to previous findings, other considerations and limitations.

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Chapter 2: Telomere length in 3-36-month-old *A. burtoni*

Introduction

Individuals in a population often vary in life-history strategies that are comprised of different components that make up an organism's life such as behaviour, rate of growth, number of offspring and body size. The pace-of-life syndrome (POLS) hypothesis suggests that behavioural traits covary with physiology and predict life history strategies on a continuum from fast to slow (Réale et al., 2010). The POLS hypothesis predicts that more bold and aggressive individuals will develop faster and die more quickly than shyer individuals. Boldness (i.e. exploration and risk-taking behaviour) has been linked to metabolism and increased capacity for locomotor activity (Binder et al., 2016). The POLS hypothesis also predicts that individuals with higher metabolic rates will grow faster on average, mature earlier, have more offspring and will have reduced longevity (Réale et al., 2010; White et al., 2016; Williams, Miller, Harper, & Wiersma, 2010). Metabolic rate and oxidative stress are closely linked to increases in ROS, similar to what is seen when animals experience stress.

Studying telomere dynamics in animal models, allows us to examine the link between survival, environmental conditions and contribute to a better understanding of life-history decisions made. Telomeres are often referred to as a 'mitotic clock' and are tightly linked to aging. Studies of the mammalian literature also demonstrate that there is a relationship between the rate of telomere loss and lifespan (Haussmann & Marchetto, 2010; Haussmann et al., 2003b). The rate of telomere change in birds has been correlated with longevity and growth (Haussmann & Marchetto, 2010). In free living great tits (*Parus major*), fledglings that hatched later than their siblings showed similar growth at first but ended with higher levels of oxidative stress and shorter telomeres; Last hatched fledglings with the highest growth rate also had the highest telomere erosion (Stier et al. 2015). Telomere length in zebra finches (*Taeniopygia guttata*) is

also associated with age, where samples taken at 25 days old were the best predictor of longevity (Heidinger et al., 2012). Telomere length in zebra finches, tree swallows (*Tachycineta bicolor*), adelie penguins (*Pygoscelis adeliae*), and common terns (*Sterna hirundo*), have been shown to decrease as age increases as would be expected from the work done in mammals (Haussmann & Marchetto, 2010; Haussmann et al., 2003b).The opposite age effect is seen in long-lived Leach's storm-petrels (*Oceanodroma leucorhoa*), where telomere length increased with age (Haussmann et al., 2003b). Elongation of telomere length with age could be due to upregulated telomerase expression to rescue tissues with critically short telomeres in more long-lived species. Reproductive investment may also alter the rate of telomere loss. Zebra finches that were allowed to breed had higher telomere loss between 25 days and 1 year than those who did not breed (Hatakeyama et al., 2016). The rate at which telomere length shortens could be influenced by environmental factors. Siskins (*Spinus spinus*) showed significantly shorter telomeres in blood and internal organs 105 days post malarial infection (Asghar et al., 2016), this could still affect the rate at which telomere length shortens and impact lifespan. Environmental factors such as stress have also been implicated in accelerating the aging process further examinations would be beneficial to address the speed at which telomere attrition occurs and its implications for organismal fitness (Asghar et al., 2016; Metcalfe & Monaghan, 2001; Simide et al., 2016; Kotrschal, Ilmonen, & Penn, 2007).

Fish, similar to birds and mammals, have shown a link to telomere length attrition with age. In medaka (*Oryzias latipes)* – a freshwater teleost with 2-year lifespan – telomere length decreased with age despite high levels of telomerase throughout the body (Hatakeyama et al. 2008). In the short-lived fish *Nothobranchius furzeri* – whose average lifespan ranges from 3-12 months – were found to have age-dependent telomere shortening in a wild derived strain despite

high levels of telomerase in skin and muscle tissue (Hartmann et al. 2009). Telomerase levels analyzed at various time points during zebrafish (*Danio rerio*) development found that telomerase levels increased during the adolescent phase but then dropped during adulthood in all tissue types except muscle (Anchelin et al., 2011). Medaka have also shown varying telomere length across different age groups; Where fish prior to 7 months showed rapid telomere attrition and fish between 7 months and 1 year had telomere growth where they then started to decline after 1 year (Hatakeyama et al. 2016). The rate of telomere attrition seems to be related to cell turnover at the time and be somewhat variable throughout the lifespan and dependent on life history. Differing tissue types have differing levels of mitotic activity; This suggests that post mitotic tissues like the brain will have relatively stable telomere length compared to tissues like skin and muscle (Gao & Munch 2015; Gopalakrishnan et al. 2013). Because the rate of telomere loss seems to be variable throughout the lifespan absolute telomere length may not be as significant as the organism's rate of loss. Cross sectional data on organism's telomere length found significant intraspecies variation in telomere length. Data provided from this study may only provide general trends. Initial data and previous studies also indicate that there may be an association between within-cohort body size and telomere length (Debes et al., 2016).

Approach and Methodology

Here, I examined different age groups of *Astatotilapia burtoni* in order to determine how telomere length changes across the lifespan. I examined four groups of fish of varying ages. These groups are comprised of six fish per group. Groups of fish that are three months, six months, twelve months and greater than twenty-four months old. This gives us a cross sectional picture of telomere length and change across the lifespan. In order to examine the telomere

length, I took samples from brain, liver and fin tissue in each fish. I took tissue from brain, liver and fin to examine the differing levels of mitotic activity in each tissue.

Subjects consisted of *A. burtoni* (N=24) maintained at 20°C ± 2°C. Subjects were bred in the laboratory and housed together in age cohorts. Subjects were fed ad libitum six days a week with commercial dried fish food. The light dark cycle is maintained at 12 hours light and 12 hours dark.

Procedure

Tissue Sampling

Standard length and weight were recorded for each fish at the time of euthanasia. Fish were placed in a container filled with TMS for ten minutes as per the standard euthanasia protocol. Sanitized surgical scissors or a new razor blade are then be used to take sample of caudal fins. Then a cut from the anus to the base of the operculum and then along the operculum is made to create a flap of tissue. The tissue flap is then pinned back, and the liver is removed. At this point samples of gonad and brain tissue is collected for both use in this thesis and possible future extension. Individual tissues are then weighed, placed into microcentrifuge tubes and be stored at -80ºC.

DNA Extraction and Quantification

Genomic DNA (gDNA) was extracted from the tissue samples using a DNeasy blood and tissue kit (Qiagen, USA) by following all manufacturer's instructions. DNA quantity was then assessed using a spectrophotometer (NanoDrop ND-1000). A working aliquot was then prepared using purified water (Milli-Q; EMD Millipore, Billerica USA) and re-checked until a final sample concentration of 5ng µl was achieved.

Telomere Length

Telomere length quantification used a modified quantitative polymerase chain reaction (qPCR) protocol based on the protocol by Cawthon (2002) and O'Callaghan, and Fenech, (2011). This qPCR protocol amplifies a DNA sequence of interest over 20-40 cycles using specifically designed primers. The repeated heating and cooling cycles unzip the double stranded DNA. Then a fluorescent molecule attaches to the unzipped DNA. Thereby allowing us to copy our gene of interest and creating a fluorescent signal that can quantify the amount of genomic copies of the telomeric repeats.

The fractional cycle number or *Cq* is the cycle number where the signal reaches a set threshold indicating that is about baseline fluorescence i.e. the threshold at which fluorescence can be detected. The *Cq* is inversely proportional to the starting quantity of DNA. This means that the higher the starting amount of DNA present the faster fluorescence is detected. Telomeric content per cell is a proxy measure for telomere length that was developed by Cawthon (2002). This method is determined as the number of telomeric repeats (T) per a number of reference gene copies (S). This allowed us to create a T/S ratio for each sample as a relative measure of telomere length and compare it to standards on each plate.

I used β- Actin as the reference gene for *A. burtoni* samples. The PCR reaction used for this study was optimized using the machine's gradient function in addition to manufacturer's specifications. Each reaction was a total of 20 μl comprised of 5μl of purified water, 10 μl of Polymerase 2X Roche Master Mix, 1 μl of forward and reverse primers (Telomere and β- Actin on separate plates based on what was being assessed) and 5μl of sample DNA. Each plate was also run with a no template control (NTC) in which case the final 5μl would be additional water or pUC at 5ng μl to simulate the complexity of a living organism. These reactions were set up and then placed into the qPCR (Bio Rad CFX machine). After extensive optimization the

reaction conditions were set to start with a 95°C hot start for 5 minutes to activate the enzyme, then denaturation at 95°C for 10 seconds, followed by a 60°C annealing for 30 seconds for 40 total cycles. After the 40 cycles, A melt curve was generated going from 45°C to 65°C with a 0.5°C incremental increase per cycle to assess the specificity of the PCR product and ensure there was no contamination.

The method used was based on work done by Cawthon (2002) and O'Callaghan and Fenech (2011). O'Callaghan and Fenech (2011) developed what they call an absolute telomere length (aTL) qPCR method which adds in a standard curve with known telomere length which is based off of values of serial dilutions of a synthesized oligomer standard that is comprised of 14 copies of the TTAGGG telomeric sequence (84bp total). Due to the manufacturer being unable to reliably ensure the oligomer standard was exactly 84bp long we opted to use the T/S ratio method. Standard curves of 5 serial 1:10 dilutions ranging from 0.05pg to 0.00005 pg of DNA were still included on every plate to standardize between plates and calculate amplification efficiency. Standard curves were generated with Bio-Rad software and PCR efficiency (E) is calculated using the formula $E = 10^{(-m^{-1})}$, with m being the slope.

qPCR has a variety of advantages when compared to other methods of Telomere length analysis. Allowing primers to better match to the telomeric sequence than to the primers themselves. A few such examples are the speed and throughput allowed. qPCR allows for real time detection of fluorescence and removes the need to run samples on an agarose gel in addition it allows for a large number of samples to be processed at once. Other methods such as terminal restriction fragmentation (TRF), which was the original technique developed for determining telomere length and is often described as being the 'gold standard' for telomere length measurement, requires the DNA to be digested with cutting enzymes that only recognize sub

telomeric regions of the DNA and then cutting the intact telomeres from all the chromosomes (Montpetit et al, 2015). These telomeres are then run on an agarose gel. Using this method can introduce subjectivity as they can be assessed differently when comparing it to a DNA ladder of known DNA fragment lengths. Quantitative Fluorescence in situ Hybridization (Q-FISH) is another method that is performed by assessing metaphase chromosomes or interphase nuclei following hybridization and labelling with a fluorescent probe. In this case, you need active cells rather than DNA and is quite labor intensive (Montpetit et al, 2015). qPCR allows for the highest sample yield while maintaining as much objectivity and accuracy as possible.

Data Analysis

All statistical analyses were carried out using R (R version 3.4.2, The R Foundation for Statistical Computing). Alpha level for significant *p*-values were set at 0.05. Normality assumptions were tested using a Shapiro-Wilk normality test and visual inspection of quantile-quantile (Q-Q) plots in addition to histograms of the residuals, and residuals against fitted values. For data that did not meet the assumption of normality a Box-Cox transformation using the Box-Cox method (Box & Cox, 1964). A one-way ANOVA was used to examine the differences in telomere length between age groups.

Telomere Length

For each sample obtained the qPCR machine calculates a fractional cycle number (Cq or Ct). This is the point at which the signal reaches a set threshold above baseline fluorescence is determined. This gives a Cq value of the sample which is inversely proportional to the starting amount of the initial DNA the telomeric repeats. This Cq values are compared using the single gene copy (SGC) that is present on every plate. Using the Cq values of the Telomeric repeats and the SGC I determined the T/S ratio by simply dividing the two numbers. Standard curves

generated by PCR machine software (Bio-Rad), and PCR efficiencies (E) were calculated as $E = 10^{(-m^{-1})}$ with *m* being the slope.

Body Condition

Weight and length measurements taken at the time of euthanasia were used to determine size and body condition. Body condition was calculated by taking the residuals obtained from the logarithm of weight and length variables.

Results

In order to test the hypothesis that a difference in telomere length across different age groups exists, a one-way ANOVA was conducted to determine the effect of age on relative liver and fin telomere length. These data did not meet the criteria for the assumption of normality, so a Box Cox transformation was applied (Box & Cox, 1964). Prior to transformation the Shapiro results were (W = 0.62, p= 0.000) for liver telomere length and (W = 0.59, p= 0.000) for fin. After performing the transformation (W = 0.96, p-value = 0.37) for liver and (W = 0.92, p-value = 0.05) for fin.

There was a significant effect of age on the relative fin telomere length in *A. burtoni,* $F(1,9) = 8.11$ p=0.01 (*Figure 2.0*). There was no significant effect of age on relative liver relative telomere length in *A. burtoni,* $F(1,9)= 0.44$ $p = 0.52$ (*Figure 2*). Sex ($F(1,9)= 2.48$ $p = 0.14$) it had no significant effect on fin relative telomere length or significant interaction with age on fin telomere length ($F(1,9)=1.55$, p=0.24). Sex also did not provide significant evidence for an effect on age in liver telomere length ($F(1,9)= 0.14$, p= 0.71) or have a significant interaction with age ($F(1,9)= 0.10$, p= 0.75).

There was no significant relationship between fish age and telomere length in liver or fin tissue for male fish (r = -0.43, p-value = 0.39, r = 0.27, p-value = 0.61). For female fish there was no correlation between liver telomere length and age (r = -0.32, p-value= 0.44). There was a correlation for female fish between age and fin telomere length (r = 0.72, p-value = 0.045). Welch's two sample t test was conducted and found no significant difference between sex and relative telomere length in fin or liver tissue. There was also no evidence of a difference in relative liver or fin telomere length between male and female fish respectively There was no significant difference in relative fin (*Figure 2.1;*t (8.81) = -0.19, p-value = 0.85) or liver telomere length (t (7.32) = 0.24, p-value = 0.81*)* between male and female fish in 12 and 36 month old fish.

Residual body condition did have a significant effect on fin relative telomere length (F(1,9)= 6.21, p = 0.03) where an increase in residual body condition was associated with increased fin telomere length. Residual body condition did not show any evidence of an effect on liver telomere length (F(1,9)= 0.01, p = 0.70). Residual body condition was not significantly related to fin telomere length, r = .09, p = 0.64 (two tailed) or to relative liver telomere length, r = -.02, p =.92 (*Figure 2.2*). Residual body condition did not differ between sexes (*Figure 2.3, t* (11.86) = 1.45, p-value = 0.17). For female fish there was no significant relationship between residual body condition and liver telomere length r = .07, p-value = 0.86 or to fin telomere length r = 0.32, p-value = 0.44. Male fish also did not show any significant relationship between residual body condition and telomere length in liver or fin tissue (r= -0.57, p-value = 0.23, and r = 0.72, p-value = 0.10).

Discussion

In the current study I found that older fish have higher fin relative telomere length than younger fish. This is similar to the results found by Haussmann et al. (2003b) where Leach's storm-petrels telomere length increased with age, the opposite of what is seen in other birds and mammals. Some *Astatotilapia burtoni* are aggressive and territorial so fin damage is likely to occur over the course of the fish's lifetime, both from direct attacks and general wear, which could lead to upregulation of telomerase (Anchelin et al., 2011). Up regulation of telomerase activity has been seen in zebrafish after caudal fin amputation. However, this upregulation in zebrafish seems to decrease after a critical point at which the telomerase seems to get less efficient, at 24-30 months (Anchelin et al., 2011). It is possible that this decrease in telomerase efficiency and telomere length could still be seen if the sampling period was closer to the end of the lifespan. The maximum lifespan of a zebrafish is only 2-3 years in captivity whereas *A. burtoni* can live up to 6 years. River sturgeon were found to have telomere length in fin tissue decrease with age across an 8-year sampling period (Simide et al., 2016). Suggesting that longer sampling periods could provide a more complete picture of telomere dynamics.

Liver tissue has lower cell turnover than fin tissue, so it is expected that the telomere length to be relatively more stable compared to fin tissue. This difference in cell turn over level and the higher level of telomerase expression in fish could be one factor in why we see no effect of age on liver relative telomere length (*Figure 2.0*), telomerase activity has been closely correlated to telomere length; if telomerase is present in the cell it can build up telomeres, however it is often not present in somatic cells of humans (Chan, & Blackburn, 2004). Telomerase has been shown to be expressed throughout life in all types of tissue in medaka (Hatakeyama et al., 2008). In

zebra finches' telomerase activity in muscle, liver and gonad tissue has been found to increase later in life (Haussmann et al., 2003). High telomerase expression has been seen in all tissue types in lobsters and this has been linked to indeterminate growth and longevity (Klapper et al., 1998). It is possible that this variable growth pattern could play a greater role in why we do not see similar telomere patterns in fish as we do in humans.

In this study we found no relationship between age and telomere length in males. Possibly due to a reduced sample size resulting in low power. Variation resulting from males' ability to switch between territorial and non-territorial phenotypes, may also affect telomere length dynamics adding variation to the data. The non-territorial phenotype devotes less energy to reproduction and territory defence and more towards somatic growth. Territorial males have bright coloration and mature testes, leaving less energy to be placed into growth, which has been suggested as a possible source of oxidative stress leading to faster telomere attrition (Metcalfe & Monaghan, 2001). I did find a correlation between fin telomere length and age in females, contradictory to what other studies have found. Female medaka have longer telomeres than their male counterparts in liver tissue (Gopalakrishnan et al., 2013). Telomerase activity in Medaka liver tissue was also higher in females than in males at 4 - 8 months old where it seemed to peak. The data from the present study does seem to follow the same general pattern as medaka where female liver relative telomere length is highest in the 6-12-month range while not significantly. Male Wistar rats (*Rattus rattus*) at 15 months of age were also found to have shorter telomeres than females in kidney, liver and pancreas tissue (Cherif, Tarry, Ozanne, & Hales, 2003). Water python (*Liasis fuscus)*, show similar results as found in this thesis, where hatchlings had shorter telomeres than older individuals however longitudinal analysis showed that telomere length did eventually decrease but at a highly variable rate (Ujvari & Madsen, 2009). Water python females

also have been shown to have longer blood telomere length than males (Ujvari & Madsen, 2009). The results from this thesis seem to suggest that with increased power, tissue specific sex differences in telomere length may found.

There was also no significant relationship between residual body condition and telomere length in either sex. While there are specific differences in metabolic profiles between the two sexes it does not appear that there was a difference in body condition. If there was a significant difference in body condition, we could expect a difference in telomere length because environmental stress and infection has been shown to increase telomere attrition (Asghar et al., 2016; Metcalfe, & Monaghan, 2001; Simide et al., 2016). There is some evidence to suggest that reproduction can cause significant sex-specific increases in telomere attrition (Hatakeyama et al., 2016; Kotrschal, Ilmonen, & Penn, 2007; Plot, Criscuolo, Zahn, & Georges, 2012). *A. burtoni* females are mouthbrooders, which involves significant investment in the form of food deprivation while raising their young inside their mouth cavity. This maternal investment can cause changes in morphology, circulating hormone levels (Grone et al., 2012; Gao & Munch 2015) and has also been linked to increased oxidative stress (Alonso-Alvarez, 2004). If any of the females in the present study had broods of fry, I would expect that we would have seen these effects. The rate of telomere attrition seems to be related to cell metabolism at the time and be somewhat variable throughout the lifespan and dependent on life history. The current study gives us a general trend of telomere length and examination in future studies should examine more sex specific effects in relation to telomere length and body condition to provide a more ecologically relevant picture.

		Telomere	ß-actin
Slope	Mean ± SE	-3.3755 ± 0.0515	-3.2145 ± 0.0115
	Range	-3.324 – -3.427	-3.203 - -3.226
y-intercept	Mean ± SE	34.578 ± 0.107	33.6515 ± 0.0235
	Range	34.471 – 34.685	33.628-33.675
R^2	Mean ± SE	0.9655 ± 0.0005	0.9985 ± 0.0005
	Range	0.965 – 0.966	0.999-0.998
PCR efficiency	Mean ± SE	97.85 ± 2.05	104.7 ± 0.5
	Range	99.9 – 95.8	104.2 – 105.2

Table 2.1. Standard Curve Summary of standard curve characteristics for Telomere and ß-actin used in study 1 on *A. Burtoni*

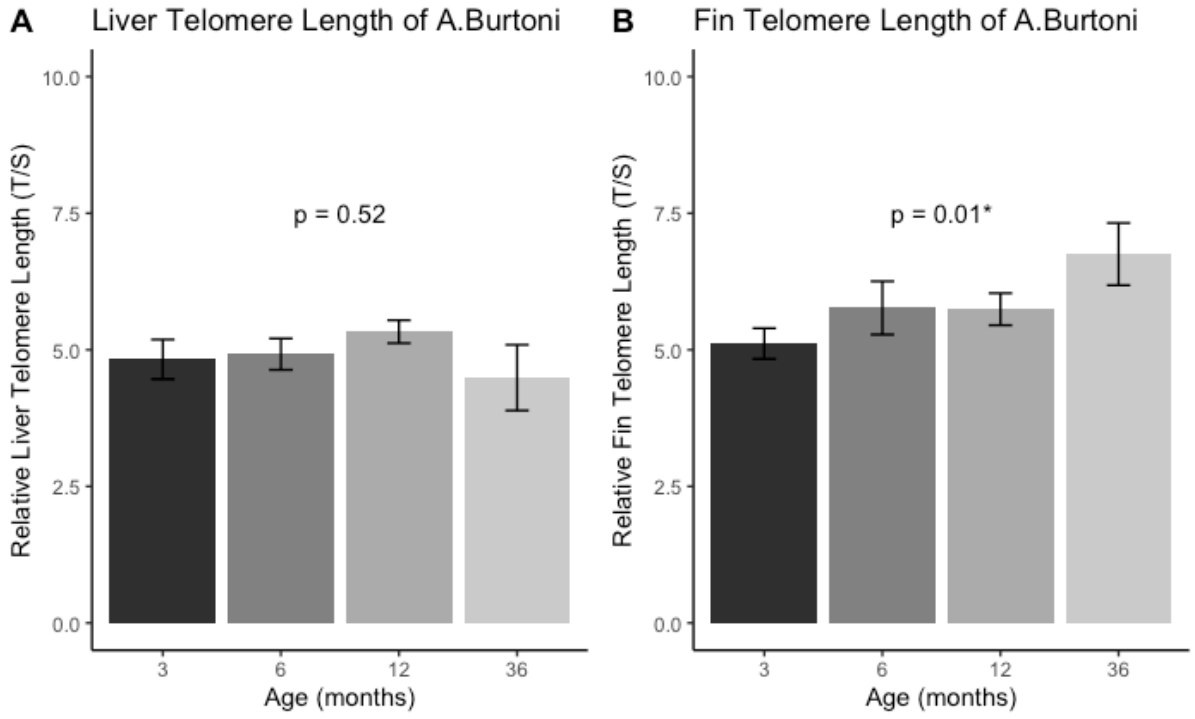

Figure 2.0. A) Relative liver telomere length per β- Actin copy in 3 – 36-month-old *Astatotilapia burtoni* $F(1,9)= 0.44$ $p = 0.52$. B) Relative fin telomere length per β- Actin copy in 3 – 36-month-old *A. burtoni* $F(1,9)= 8.11$ $p = 0.01$.

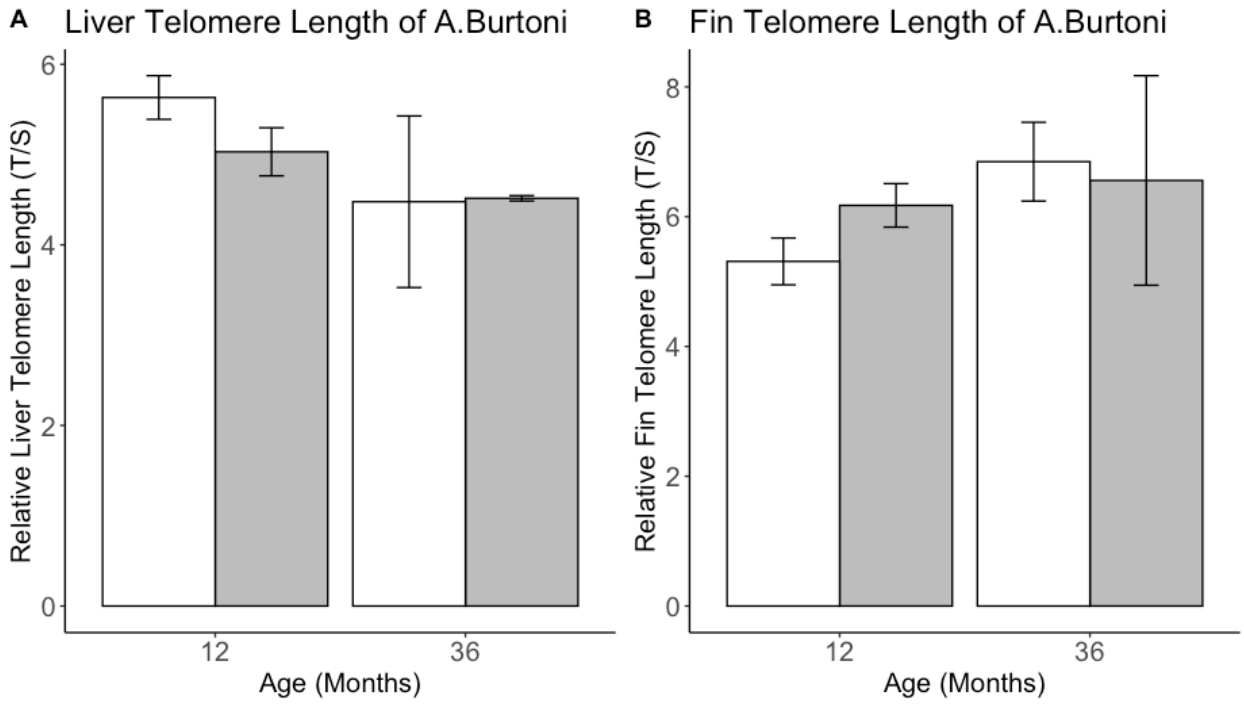

Figure 2.1. Relative Liver and Fin telomere by sex of the fish in the 12- and 36-month group. White bars indicate females and grey males. There was no significant difference in relative fin or liver telomere length between male and female fish respectively (t (8.81) = -0.19, p-value = 0.85, t (7.32) = 0.24, p-value = 0.81).

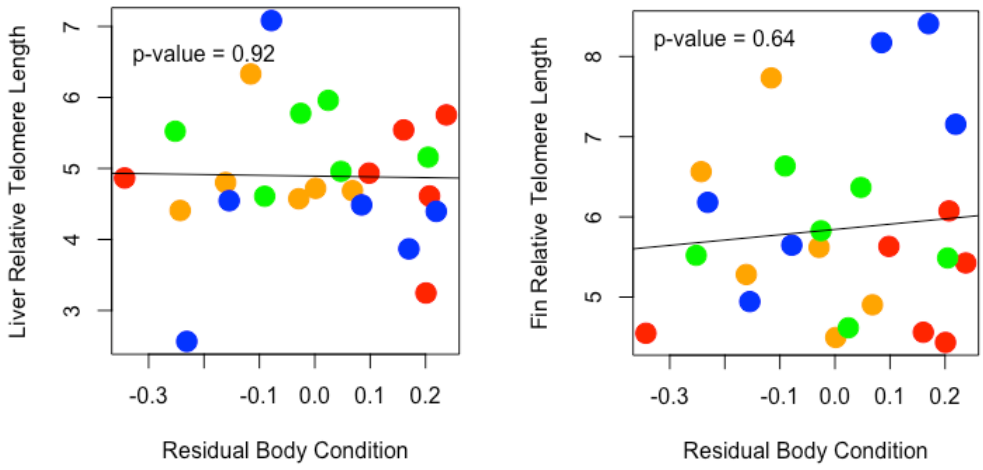

Figure 2.2. Red indicates three months, orange six, green twelve and blue twenty-four months. Residual body condition was not significantly related to fin telomere length, $r = .09$, $p = 0.64$ (two tailed) or to relative liver telomere length, $r = -.02$, $p = .92$.

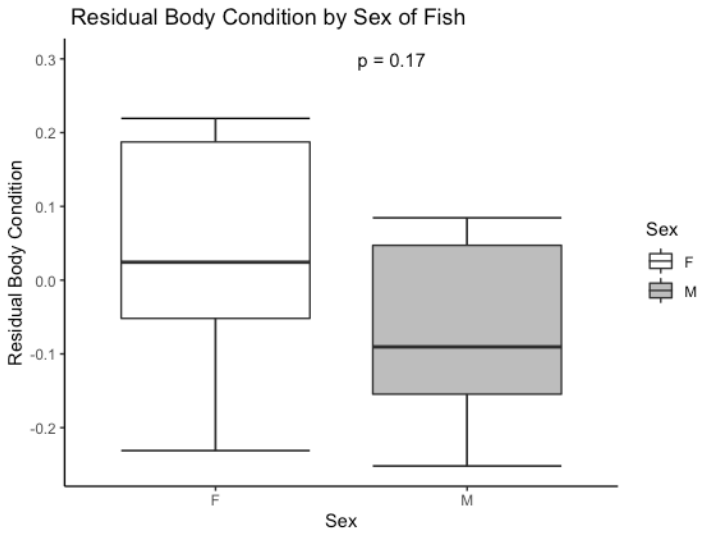

Figure 2.3. Residual body condition of the fish by gender. Residual body condition did not significantly differ between sexes ($t(11.86)=1.45$, p-value = 0.17) .

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Chapter 3: Telomere length and Early Life Stress in *A. nigrofasciata*

Introduction

Early life conditions can have significant effects on both the long- and short-term fitness of an organism (Watson, Bolton & Monaghan, 2015). Early life is a critical period for brain development and exposure to stress during this time can be particularly influential on an organism's phenotype and future risk of disease (Entringer, de Punder, Buss, & Wadhwa, 2018; Watson, Bolton, & Monaghan, 2015). Stress occurring in early life has been suggested as a mechanism that transfers information about the environment into physiological changes that affect the health and longevity of an organism (Epel et al., 2004; Beery et al., 2012). Increases in glucocorticoid levels have been shown to increase metabolism and DNA damage (Monaghan, & Ozanne, 2018).

Telomeres, non-coding DNA sequences found at the end of chromosomes, can potentially show us how an organism's early life connects to their long-term fitness outcomes (Watson, Bolton & Monaghan, 2015). In human cells, telomeres shorten by about 30 – 200 base pairs per cell division. Approximately 10 of these base pairs are thought to be lost because of this 'end replication problem', the remaining loss is partly due to reactive oxygen species (ROS) (Dijkstra, Pierotti, Seehausen & Metcalfe, 2016; Epel et al., 2004; Monaghan, & Ozanne, 2018). Telomeres can become mutated or broken when ROS – a by-product of metabolism – build up in the cell. Telomeres protect the ends of chromosomes from this damage and this is possibly why they are highly conserved across eukaryotes (Monaghan, & Ozanne, 2018). Recent evidence suggests that social stress may also shorten telomeres in humans by increasing oxidative stress caused by the release of cortisol (Epel et al., 2004). Herborn et al. (2014) found that a group of long-lived birds had telomere attrition that was significantly accelerated after early-life stress and

could be leading to reduced longevity. However, some stress during this time could provide some positive consequences leading to better coping as the organism ages (Monaghan, 2014).

The introduction of stress in early life has also been associated with compensatory or 'catch-up' growth after the stress has subsided (Metcalfe, & Monaghan, 2003; Monaghan, & Haussmann, 2006). One-year-old female tree swallows with shorter than average telomeres have lower survival rates than those with longer telomeres (Monaghan, & Haussmann, 2006). This suggests that telomere length effects exist early in life, and that telomere shortening is linked to early growth conditions and the pace of deterioration later in life. In addition, Jennings, Ozanne, Dorling, and Hales (1999) demonstrated that in the postnatal life of rats, growth retardation was associated with longer kidney telomeres; however, growth retardation in foetal life was associated with 'catch up' growth and shorter average telomere lengths. Results from Hofmann, Benson, and Fernald, (1999) suggest that growth rate can be altered quickly in the face of environmental change, specifically in males. It has also been suggested that African cichlids can alter growth trajectories when there are changes in the social hierarchy (Heg, Bender & Hamilton, 2004). Together these results suggest that both environmental stress and changes in social hierarchy can alter life-history trade-offs and therefore growth rate. Individuals who are exposed to stress are more likely to experience catch up growth after these environmental changes occur (Lee, Monaghan, & Metcalfe, 2012). Males are suggested to exhibit catch up growth in relation to dominance and social changes which in turn may increase oxidative stress. POLS also suggests that bold-shy behavioural traits covary with life history trade-offs on a slow-fast continuum. Where bolder individuals who assert dominance and are more aggressive will die at a younger age than shyer individuals. The POLS hypothesis predicts that this is because more resources are diverted to these high energy behaviours. Impacting their metabolism and oxidative

stress levels. The ability to make these trade-offs in relation to social status suggests that growth rate is relatively plastic and involves increased resources and metabolic process. Increasing these metabolic processes have been shown to decrease telomere length and longevity for these animals (Campisi, Kim, Lim, & Rubio, 2001; Haussmann et al. 2003; Haussmann, & Marchetto, 2010). In *A. Burtoni,* dominant fish showed higher levels of reactive oxygen metabolites when compared to subordinate fish (Border, et al., 2019). Dominant males have bright coloration and defend spawning territory with aggressive behaviour that is tightly linked to reproductive ability. Maintenance of social status upregulates the reproductive axis and leads to increased metabolic rate (Dijkstra, Seehausen, & Metcalfe, 2013). Suggesting that maintaining social dominance could be a potential trade-off between longevity and reproduction. Stress from defending territory, investing in growth or reproduction may increase oxidative damage or may be maintained but take resources away from antioxidant defence or telomere maintenance leading to delayed damage and telomere length attrition.

In animals, short-term or moderate stress can promote more adaptive stress coping, such as increased survival in the presence of a predator or aggressive conspecific. Increased stress has been shown to reduce social interaction in animals where these relationships are beneficial for predator avoidance (Beery, & Kaufer, 2015; Taborsky, Arnold, Junker, & Tschopp, 2012). Studies have shown that the teleost stress response system – the hypothalamus–pituitary–interrenal (HPI) axis – follows a similar neuroendocrine control pattern as mammals and other terrestrial species, making them a convenient model for studying stress and coping (Aluru, & Vijayan, 2009; Wendelaar Bonga, 1999c). Both human and teleost telomere length, (specifically the Japanese medaka, a close relative of *A. burtoni* and *A. nigrofasciata*) has been found to be on average 10kb (Au et al. 2009). While murine models generally have telomeres that are 50 to

150kb (Calado, & Dumitriu, 2013). I used the model fish species *Amatitlania nigrofasciata*, a Central American cichlid to investigate the effects of early life stress on telomere length, and growth rate.

Approach and Methodology

Subjects consist of lab bred *A. nigrofasciata* (N= 23) maintained at 20°C ± 2°C. Fish were bred in the lab and housed separately with clear dividers between them to avoid fin damage. Subjects were fed ad libitum six days a week with commercial dried fish food. The light dark cycle is maintained at 12 hours light and 12 hours dark.

I randomly divided each of the 2 broods after they become free swimming into a predator stress group, and a control group to eliminate the potentially confounding variables of genetic background and maternal effects. The predator stress group is chased with a net for 2 minutes every day for 2 weeks. The stress group was chased at four months of age to ensure they were of sufficient size to obtain enough fin tissue to perform the qPCR assay. Additionally, to ensure that this was prior to sexual maturity and still in the early-life stress critical period. These sorts of simulated predation stresses are known to increase cortisol production, slow growth and alter time of breeding (Pollock, et al., 2005; Ramsay et al., 2009; Moscicki, & Hurd, 2015). The control group is housed in identical conditions without the net chase treatment. Apparatus used are the submerged plus maze and open field task. These behavioural assays are adaptations of classic apparatus used in rodent models and have been adapted for use in fish. Confirmation of the task's validity has been confirmed using anxiolytic drugs (Hope, Hamilton, & Hurd, 2019a).

Water-borne hormone samples, which measure cortisol released across the gills, was used as a non-invasive proxy for blood hormone titers (Earley et al., 2006; Kidd et al., 2010; Sebire, Katsiadaki, & Scott, 2007). Each fish was sampled individually before and after the treatment period. In order to obtain as close to a baseline level as possible each fish was sampled three

times prior to baseline test to habituate to the confinement stress. I then obtained a baseline cortisol level as well as a physiological proxy with which to demonstrate that our treatment produces "stress". These hormone samples were then analysed using a solid phase extraction method with C18 columns. Columns are frozen until cortisol ELISA.

Both before and after stress exposure, fin clips were collected for telomere length assays, and weight and length measures were taken to assess growth rate. I took fin clips (approx. 1mm ^{2}) one week before stress treatment and one week after and then again one month later at the time of euthanasia to determine change in telomere length. Fin clips taken from the right paired pelvic fin and at second sampling the left as they are assumed to have comparable telomere lengths (Näslund, et al., 2015).

Genomic DNA was extracted from the tissue samples using a DNeasy blood and tissue kit (Qiagen, USA) by following all manufacturer's instructions. DNA quantity was then assessed using a spectrophotometer (NanoDrop ND-1000). A working aliquot was then prepared using purified water (Milli-Q; EMD Millipore, Billerica USA) and re-checked until a final sample concentration of 5ng µl was achieved.

Telomere length quantification are done using a modified quantitative polymerase chain reaction (qPCR) assay based on the protocol by Cawthon (2002) and O'Callaghan, and Fenech, (2011). Oligo-primers designed with minor sequence mismatch to avoid primer dimerization are used to amplify telomeric sequence. A synthesized 84 bp HPLC purified telomeric sequence template of known concentration is used in a 1:10 dilution series to establish the standard curve, as well as confirm primer efficiency. This standard curve is used to calculate telomeric sequence content per sample in kb. A single copy gene (SCG) is used to normalize input template amount

across samples as a means to standardize genome copies per sample (O'Callaghan, & Fenech, 2011).

Procedure

Submerged Plus Maze

The plus maze is comprised of an "+" configured maze with black inserts that can be inserted into two of the maze arms (*Figure 3.1;* Hope et al., 2019b). The black arms of the maze face the experimenter and the apparatus is filled with 11 cm of water. The maze is lit from above with a 30w fluorescent bulb and recorded for 300 seconds from above by a webcam attached to a laptop. The experimenter is hidden from the fish's view by a curtain while the trial is in progress. The Plus maze is used as a behavioural assay to determine anxiety response levels in animal subjects. Fish are scored by the number of total lines crossed throughout the entire 300 second trial and the total number of lines crossed in dark and clear arms of the maze up to their opercula. The fish are also scored on the time from entering an arm of the maze up to the opercula until they exit the arm past the opercula. The proportion of the 300 seconds that are spent in the open arms compared to dark arms is also calculated.

Open field

The open field apparatus consists of a 40 L tank (26.5 cm X 51 cm X 31 cm) filled with 11 cm of water (*Figure 3.2*). Underneath the tank is a plastic sheet on which a 5 x 10 grid with 5 cm x 5 cm squares drawn on it. This grid covers the entire bottom area of the tank. In the middle of the grid is a circle on which an acclimatization enclosure is placed. Acclimatization occurs in a piece of 9 cm diameter tubing cut to 6.5 cm tall attached to an 11 cm x 11 cm white plastic tile. The acclimatization tubing is attached to a string so it could be lifted from behind a curtain. Acclimatization lasts for 120 seconds. Trials are recorded for 300 seconds from above by a webcam (Moscicki & Hurd, 2015). The open field apparatus is used to test locomotor activity

and willingness to explore novel environments. Videos of the fish are scored using the total number of seconds from when the fish enters a corner, outside or inside square up to the opercula until they exit the square past the opercula. Fish are also scored on the number of each type of squares crossed up to the opercula and total number of squares crossed during the entire 300 second trial.

Hormone Collection

Hormone samples are collected from fish before and after testing to ensure measure accurate changes in levels of cortisol. Three initial samples were collected to mitigate confinement stress and obtain an accurate baseline. Samples are obtained by placing fish in a beaker sanitized with 100% ethanol, then rinsed with distilled water and filled with 50mLs of distilled water. Fish were placed in the beakers using a sanitized dip net and then were surrounded by barriers to prevent additional stress of seeing other fish or the experimenter. The fish were left in the beaker for 30 minutes to ensure that there is enough urine to process. Between samples nets are sanitized with 100% ethanol and rinsed with distilled water to prevent contamination between samples. These hormone samples are analysed using a solid phase extraction method with C18 columns (Bond Elut 200 mg 3 mL, Agilent), a 20-port vacuum manifold (VM20, Sigma-Aldrich) and a vacuum. I activated columns with 100% methanol followed by distilled water prior to sample loading. Columns are stored frozen until a cortisol ELISA is performed to quantify hormone levels.

C18 columns were taken out and brought up to room temperature for approximately 30 minutes. Then placed into the top of a Chromabond Vacuum manifold. 4mL of ethanol was pipetted into each C18 column. The ethanol is then vacuumed through the column into labeled 100mm glass test tubes placed below. Samples then are mixed thoroughly and then split in half to ensure consistency and sufficient amounts for the ELISA protocol. The samples are then

placed in a hot water bath at 37°C and dried under a constant stream of nitrogen. Samples were stored at -20°C until the day of the ELISA protocol. The day of the ELISA protocol samples are resuspended using 95% ELISA buffer and %5 EtOH. The assay is then preformed using the manufacturer's protocol.

Tissue Sampling

Fin clips taken before and after the stress treatment were taken from the paired pelvic fins (approx. 1mm^2) to avoid increasing the amount of telomerase expressed in the tissues. First sampling was taken from the right pelvic fin and then the next was taken from the left. Tissue was taken from paired pelvic fins because they are assumed to have comparable telomere length. In addition, pelvic fins are used in a more static manner for fine tuning movements and keeping the fish level in the water so removal of one of these pelvic fins seems to have little to no effect on their ability to swim and remain level. A new scalpel blade or sanitized surgical scissors was used for each sample. The tools being used to collect the samples were sanitized with 96% ethanol followed by a rinse with distilled water to remove DNA contamination and to avoid cross contamination between samples/fish. An anesthetic bath was prepared using tank water and TMS (150 mg/L for induction) including a buffer. Anesthetized fish were then transferred to a clean petri dish. The fin was then clipped using scalpel or surgical scissors and the sample was placed in microcentrifuge tubes on ice until they could be immediately moved to and stored at -80ºC.

Standard length and weight were recorded for each fish at the time of euthanasia. Fish are be placed in a container filled with TMS for ten minutes as per the standard euthanasia protocol. Sanitized surgical scissors were then used to take a fin sample. Then a cut was made from the anus to the base of the operculum and then along the operculum to create a flap of tissue. The flap tissue is then pinned back, and the liver is removed. At this point gonad, and brain tissue are

collected for both use in this thesis and possible future extension. Individual tissues are then weighed, placed into microcentrifuge tubes and be stored at -80ºC.

DNA Extraction and Quantification

Genomic DNA was extracted from Fin clipped tissue using a DNeasy blood and tissue kit (Qiagen, USA) by following all manufacturer's instructions. DNA quantity was then assessed using a spectrophotometer (NanoDrop ND-1000). A working aliquot was then prepared using purified water (Milli-Q; EMD Millipore, Billerica USA) and re-checked until a final sample concentration of 5ng µl was achieved.

qPCR

Examination of the DNA samples is accomplished using a Maxwell 16 robot (Promega). Telomere length quantification is done using a modified quantitative polymerase chain reaction (qPCR) protocol based on the protocol by Cawthon (2002) and O'Callaghan, and Fenech, (2011). This qPCR protocol amplifies a DNA sequence of interest over 20-40 cycles using specifically designed primers. The repeated heating and cooling cycles unzip the double stranded DNA. Then a fluorescent molecule attaches to the unzipped DNA. Thereby allowing us to copy our gene of interest and creating a fluorescent signal that can quantify the amount of genomic copies of the telomeric repeats. This method was chosen because telomeres are repeats of TTAGGG they are particularly prone to primer dimerization. The method developed by Cawthon (2002) overcame this problem by developing primers that bind to the c and g rich segments but leaving small mismatches. Allowing primers to better match to the telomeric sequence than to the primers themselves. The amount of telomeric product produced from this procedure is usually compared to a single gene copy as a means to develop a T/S (Telomere/Single gene copy) ratio.

The protocols used in this study comply with Canadian Council on Animal Care guidelines and have been assessed and approved by the University of Alberta Biological Sciences Animal Policy and Welfare Committee (protocol number 00000055).

Data Analysis

All statistical analyses were carried out using R (R version 3.4.2, The R Foundation for Statistical Computing). Alpha level for significant *p*-values were set at 0.05. Any variables that did not meet the assumption criteria were transformed using the Box- Cox method (Box & Cox, 1964). These measures were assessed for normality using a Shapiro-Wilk test. Levene's test was also performed to assess the equality of variances on all variables. One fish was excluded from the analysis because of incomplete values. I assessed all variables for normality and transformed using a Box-Cox transformation if they did not meet this assumption. I then conducted a principal component analysis using the prcomp() function in R to ensure parsimony and remove colinear variables. One time-related variable was removed from the analysis to remove collinearity created by the time variables summing to 300seconds. A Kaiser-Meyer-Olkin measure verified that all measures and the sampling adequacy for the analysis was 0.5. I then analysed the eigen values and 3 dimensions had values above Kaiser's criterion of 1. These behavioural components were then used in subsequent models. Group differences were assessed using a student's t-test and a two-way ANOVA.

Behavioural Assays

The behavioural assays used gather measures of locomotor behaviour in the form of total lines crossed up to the opercula in the plus maze and total number of squares crossed up to the opercula in the open field task. In addition, I measured the total amount of time fish spent in specific areas of these tasks to indicate proactive versus reactive coping styles. I made ratio variables to look at if the proportion of behaviours varied between tests. These were made in R

by dividing the variable by the total sum. I then made delta variables to look at the change in the amount of a behaviour between the first and second test by subtracting the second test variable from the first in order to examine the change between tests (See *Table 3.1*).

Growth rate

All cohorts weight and standard-length measurements was assessed for normality using a Shapiro-Wilk test. Any variables that did not conform to normality were assessed to determine which transformation is most appropriate to return data to normality. Calculations are then be performed to determine the fry's growth over the course of the study. Basic growth rate is calculated by taking the weight and length measurement most recently attained and subtracting it from the initial first weight and length then dividing by the initial weight and length ($Inital - Last \div Last$). Growth rate per time interval is determined using the formula: growth rate = (initial / last)$^{1/n}$ – 1, where n = the number of time periods.

Telomere Length

For each sample obtained the qPCR machine is calculate a fractional cycle number (Cq or Ct). This is the point at which the signal reaches a set threshold above baseline fluorescence is determined. This gives a Cq value of the sample which is inversely proportional to the starting amount of the initial DNA the telomeric repeats. This Cq value is normalized using the single gene copy (SGC) that is present on every plate to normalize the obtained values. Using the Cq values of the Telomeric repeats and the SGC to determine the T/S ratio by simply dividing the two numbers. Standard curves are generated by PCR machine software (Bio-Rad), and PCR efficiencies (E) calculated are calculated as E=10($-m-$1) with m being the slope.

Results

PCA

A principal component analysis was conducted on the behavioural measures using the prcomp() function in R. This was used to ensure parsimony by using the smallest number of

explanatory variables and to combine or remove any variables that are colinear. A Kaiser-Meyer-Olkin measure verified that all measures and the sampling adequacy for the analysis was 0.5. I then analysed the eigen values and 3 dimensions had values above Kaiser's criterion of 1. One of the time related variable was removed for each task due to the total summing to 300 seconds. The first two components were retained. Component one accounted for 40.1% of the variance and was characterized by change in lines crossed in clear arms and change in percent of time in clear arms and negatively with change in lines crossed in dark arms. Suggesting that PC1 is primarily associated with exploration and activity for the Plus Maze task. Component two accounts for 29.6% of the variance and is primarily composed of total squares crossed, and squares crossed inside in the Open field task. Suggesting that PC2 is primarily associated with thigmotaxis in the open field task. The change in the ratio of time spent in outside squares compared to inside squares before and after stress treatment was significantly related to PC1 in the open field task *(r*= -0.96, $p < 0.000$). There was also a significant relationship between the change in total lines crossed and PC1 *(r*= 0.77, $p < 0.0001$) in the plus maze. Fish that had a higher change in total number of lines crossed scored higher on PC1

The latency for fish to emerge into a novel environment was measured before and after the experimental treatment and showed no effect of stress (t(17.03) =- 0.21, p-value = 0.83). PC1 or PC2 were not significantly related to time to emerge (*r= -0.33, p= 0.1188; r= -0.10*, p = 0.62).

Cortisol

At the time of first testing fish in the control and stress groups showed similar cortisol levels (t (19.9) = -0.11, p=0.91). At the second time of testing fish in the stress treatment group only showed moderate evidence of an effect of stress t(10.18)=-2.08, p=0.06). However, there was no significant difference between groups when looking at the change in cortisol levels

between testing (t (13.23) = -1.9093, p = 0.078). Change in cortisol across the sampling period was not significantly associated with PCA scores (PCA1, r=.27, p=0.22, or PCA2, r=.24, p=0.27)

Growth rate

At the first measurement there was no significant difference between weight (t (15.01) = -1.32, p-value = 0.20) or length (t (20.95) = -1.3178, p-value = 0.20) for the groups. There was no difference in growth rate or growth interval between fish in the control group and the stress treatment group. PC1 and PC2 were both not significantly related to a change in length (PC1 r = -0.06, p = 0.79, PC2 r = -0.16, p = 0.47) or a change in weight (PC1 r = 0.089 p = 0.69). There was no main effect of growth rate on liver relative telomere length (F(1,8)=0.002, p=0.961). There was also no interaction effect with stress (F(1,8)= 0.23, p=0.64) or with either principal component (PC1, F(1,8)= 0.63, p=0.45, F(1,8)= 0.043, p=0.84).

Telomere Length

There was no significant difference between relative telomere length from terminal liver (t(12.600= -1.50, p= 0.43) and fin tissue samples (t(14.39)= 0.81, p=0.13). Liver telomere length appears to have remained stable while fin tissue is trending towards being higher than the control group (*Figure 3.3*). PC1 was not significantly related to liver telomere (r = *0.38*, p = 0.10) or to fin telomere (r= *-0.19, p = 0.40)*. *PC2* was also not significantly related to liver relative telomere length (r= 0.27, p = 0.21) or fin relative telomere length (r= -0.31, p = 0.18).

There was no significant main effect of stress on liver relative telomere length (F(1,14)= 0.97, p=0.34) or interaction effect with PC1(F(1,14)= 0.21, p=0.64) or PC2 (F(1,14)= 0.30, p=0.58).However, in fin tissue there was a significant interaction effect of stress treatment with PC1(F(1,18)=5.02, p= 0.04) and PC2 (F(1,18)=4.67, p= 0.04).

Discussion

PC1 was associated with change in both lines crossed and percent of time within clear arms. PC1 was also negatively associated with change in lines crossed within dark arms (Table 3.1). Higher PC1 scores indicate a decrease in locomotor activity between the first sampling and the second. Stressed fish that had high fin relative telomere length scored lower on PC1 indicating that stressed fish had less exploratory behaviour across sampling periods. Whereas stressed fish that that scored higher on PC2 indicating increased locomotor and thigmotaxis had lower relative fin telomere length. Control fish seemed to have relatively stable relative fin telomere length regardless of their PC1 and PC2 scores. Stressed fish that scored higher on PC1 had lower fin relative telomere length and decreased locomotor activity in clear arms which could be an indicator that their coping style is becoming more reactive and displaying less proactive coping strategies as they are being informed about their environment. Stressed fish also had a higher change in the ratio of time spent in dark arms compared to clear arms (*Figure 3.5)* suggesting that these fish adopted a more reactive coping style after stress exposure than control fish.

PC2 is primarily representative of thigmotaxis (*Table 3.1)* and stressed fish who scored higher on this measure had lower fin relative telomere length similar to what is seen in *Figure 3.6*. Taken together these fish show a behavioural difference when exposed to early life stress where fish exposed to stress show increased hiding behaviours. There was a difference in cortisol release before and after the stress treatment (*Figure 3.7*) so there was an increase in stress responsivity to the treatment. However, there seems to be a more complex interaction between these variables. Relative telomere length from terminal liver and fin tissue samples had no main effect difference between groups (*Figure 3.3*). Liver telomere length remained the same while it appears that fin tissue is trending towards being higher in the stress group than the control group,

possibly suggesting an upregulation of telomerase. Rats exposed to a 12-week stressor their blood telomerase activity in their blood (Beery et al. 2012). One possible explanation for these results can be seen from Epel et al. (2010) which suggests that acute stress may ramp up telomerase activity, whereas chronic stress weakens the telomerase system to exhaustion. Telomere loss could occur more drastically later in life when telomerase loses efficiency. The upregulation of telomerase earlier in life may masks any damage until well past sexual maturity. However, there are exceptions to this; one of the most salient examples being post-traumatic stress disorder (PTSD) where an acute exposure to stress can cause sustained effects on glucocorticoid levels and anxiety-like behaviours (Clinchy, Sheriff and Zanette, 2013). Animal models show that exposure to alarm cues or predators can confer sustained stress similar to what is seen in humans with PTSD (Cohen, Kozlovsky, Richter-Levin, & Zohar, 2010). It is possible that moderate net chasing stress is not sufficient to produce these kinds of effects in telomere length at the individual level in the time period. The amount of stress required to make permanent changes on the individual level may need to be either more intense or more chronic in nature. Additionally, greater statistical power may be required to detect these effects, requiring a larger sample size.

Telomeres are highly conserved across eukaryotes and play an important role in human cell deterioration and aging (Campisi & Robert, 2014). Critically short telomeres in humans is linked to cell death and cellular senescence where a high number of senescent cells leads to inflammation (Campisi, 2005) and various age-related diseases such as stroke (Tian et al., 2019). However, the cellular mechanism for cellular senescence and death is also an important anti-cancer mechanism. Telomerase dysfunction can lead to unchecked cellular replication where, cellular senescence or death is the cells defence against this unchecked replication (Campisi,

2005; Campisi & Robert 2014). The balance between cell deterioration or replication is vital for the health of the organism and may play a role in the pace of life where tumor suppression early in life allows for reproduction, but adversely affects longevity contributing to a faster pace of life. Evidence of this has been seen in studies of early life telomere length being associated with longevity (Heidingeret al., 2012; Eastwood et al., 2019) as well as with early-life stress being associated with reduced longevity (Monaghan & Haussman 2015). While most of these studies examine passerine models, we do see similar results in studies of sturgeon; chronic stress exposure negatively affected longevity and reproduction (Bayunova, et al., 2002) and heat stress significantly reduced telomere length (Simide, Angelier, Gaillard, & Stier, 2016). Cell arrest or cell death can also be found in cells with long telomeres caused by breaks in DNA from reactive oxygen species or environmental radiation. In these cases, cell arrest or cell death is to protect against cancer (Reddel, 2003). In mice, high levels of telomerase are also expressed in some cells where telomere lengthening can occur as well as cases of human cancer which have shown telomere lengthening (Bryan et al., 1997; Reddel, 2003). This could be similar to what we have seen in fish: stress on the organism could be upregulating telomerase to maintain length and tissue function but after stress an immune response may send the cell into arrest or death to mitigate cancer risk. Chronic levels of stress over a long period of time may also over tax the immune system leading to unchecked proliferation, cancer and death of the organism. DNA damage can also be fatal to the cell on its own, this is known as passive death due to a failure of DNA repair and proper functioning; active death is a delayed damage response brought about through the innerworkings of the cell resulting in one of three main option: the cell is repaired, the cell ceases to function or the cell dies (Borges, Linden, & Wang, 2008; Wang, 2019). The difference in these two categories is not easily discernable experimentally because of the time

delay and the implication of necroptosis, which can be activated by other factors such as oxidative stress or inflammatory cytokines (Wang, 2019) The mechanism in which the cell "decides" whether damage is irreparable or the cell is salvageable still remains unclear (Surova, & Zhivotovsky, 2013).

Early life is generally a time of intense growth, and susceptibility to environmental influences (Hofmann, et al.,1999; Doring & Hales, 1999). Individuals who have been exposed to stress during this phase have been suggested to exhibit catch up growth after these environmental changes occur (Lee, Monaghan, & Metcalfe, 2012). In the current study, we did not see any effect of treatment on growth (*Figure 3.9*), but an increased length of sampling could show an increase in growth following the stress treatment. Increasing the time span could also show more of how this compensatory growth affects telomere length. Indeterminate growth levels and increases in telomerase expression could also mask these effects if the treatment is not sufficient. The current study provides only a snapshot of the first year of *A. nigrofasciata* life span where most of the change and growth in telomere length has been purported to occur.

	PC1	PC2
Percent Variance Explained	40%	30%
Change in squares crossed inside	-0.06	0.08
Change in squares crossed outside	-0.05	0.63
Change in total squares crossed	-0.01	0.65
Change in percent of time spent in dark arms	-0.56	-0.14
Change in percent of time spent in clear arms	0.56	0.06
Change in lines crossed in dark arms	0.18	-0.38
Change in lines crossed in clear arms	0.57	0.00

Table 3.1. Principal components of change in behavioural variables in plus maze and open field task.

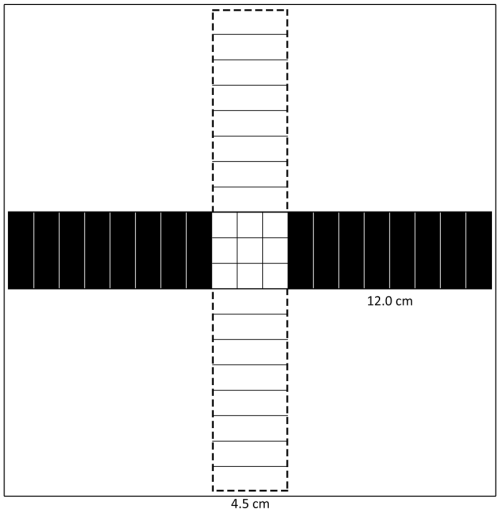

Figure 3.1. Diagram of submerged plus maze.

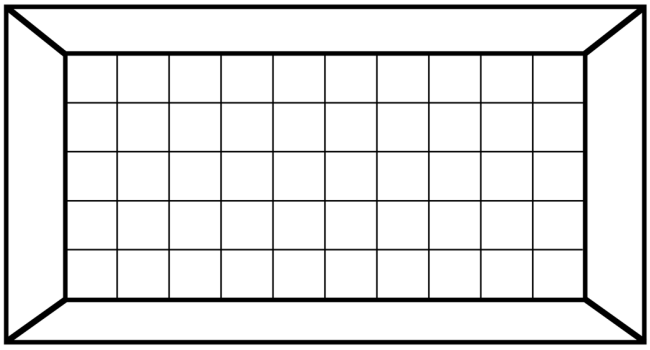

Figure 3.2. Diagram of the open field apparatus

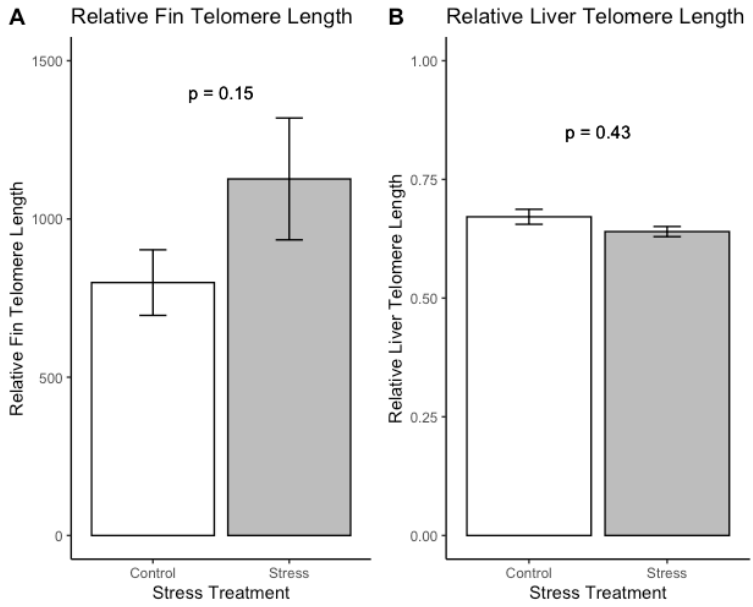

Figure 3.3. Relative telomere length from terminal liver and fin tissue samples. There was no significant difference between relative telomere length from liver tissue ($t(12.600= -1.50, p= 0.43$) and fin tissue samples ($t(14.39)= 0.81, p=0.13$). Liver telomere length remained the same while it appears that fin tissue is trending towards being higher than the control group possibly suggesting an upregulation of telomerase.

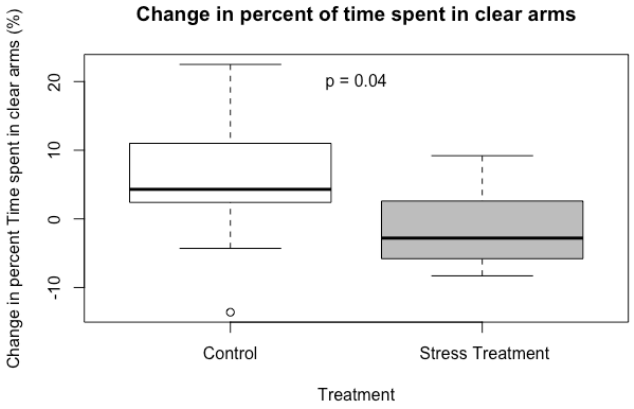

Figure 3.4. There was a significant difference in the change in percent of time spent in clear arms in the control group compared to the stress group ($t(20)= 2.18$, $p=0.04$). Higher change values suggest that fish spent more time in clear arms at the first sampling indicating more exploration.

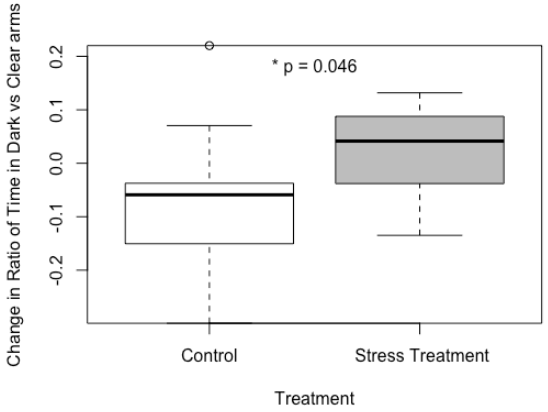

Figure 3.5. Change in the ratio of time fish spent in dark arms compared to clear arms of the plus maze before and after stress treatment. Fish in the stress treatment group had a higher change ($t(20) = -2.12$, $p= .046$) in the ratio of time they spent in dark arms of the maze compared to fish in the control group.

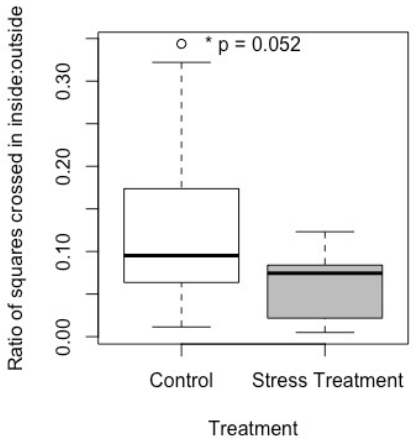

Figure 3.6. The ratio of squares crossed in inside squares in the open field task is higher (approaching significance, t(16) = 2.0903, *p = .05)* in control fish than fish in the stress treatment group

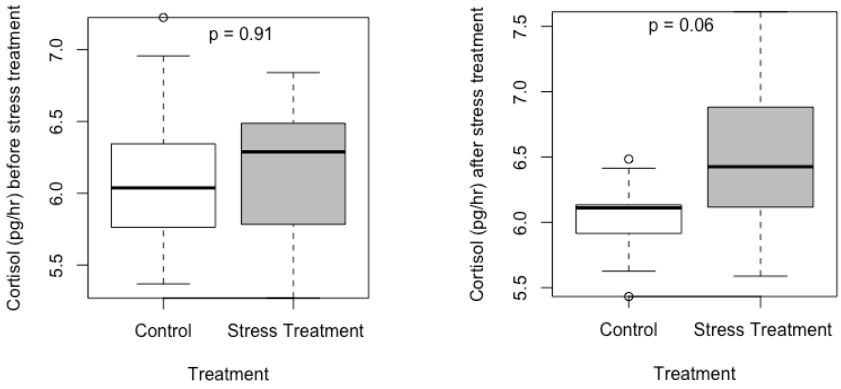

Figure 3.7. A) Cortisol measurements taken before any treatment was done and B) Cortisol measured in pg/hr after the experimental treatment. The cortisol level for the control group remained similar across both tests but cortisol release was increased in the treatment group after experiencing the stress treatment.

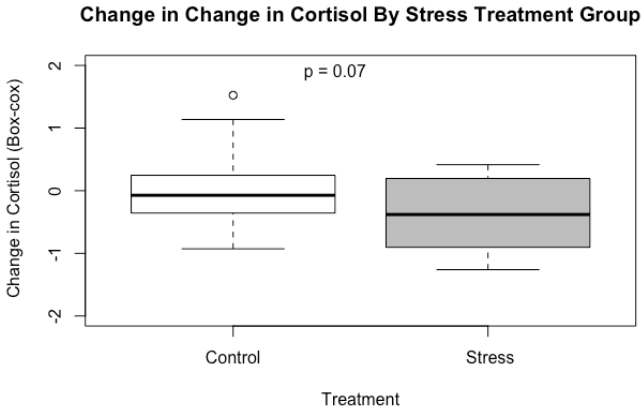

Figure 3.8. There was no significant difference between groups when looking at the change in cortisol levels between testing (t (13.235) = -1.9093, p = 0.078).

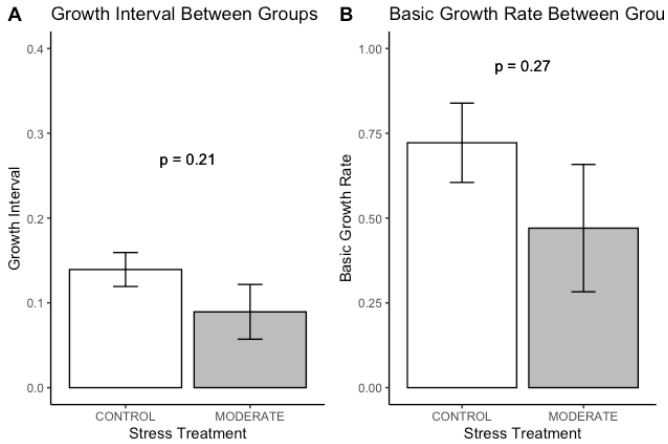

Figure 3.9. A) The difference in growth interval between the control and stress groups t (13.95) = 1.31, p = .21. Growth interval is calculated by taking the weight at the last measurement divided by the first weight measurement taken to the power of inverse ¼. (weight4 / weight1) $^{(1/4)}$ $^{-1}$. B) The difference between the basic growth rate of fish between the stress and control groups t(14.01) = 1.14, p = .27. Basic growth rate is calculated by taking the last weight measurement subtracted by the first measurement divided by the initial weight measurement. (weight4-weight1)/weight.

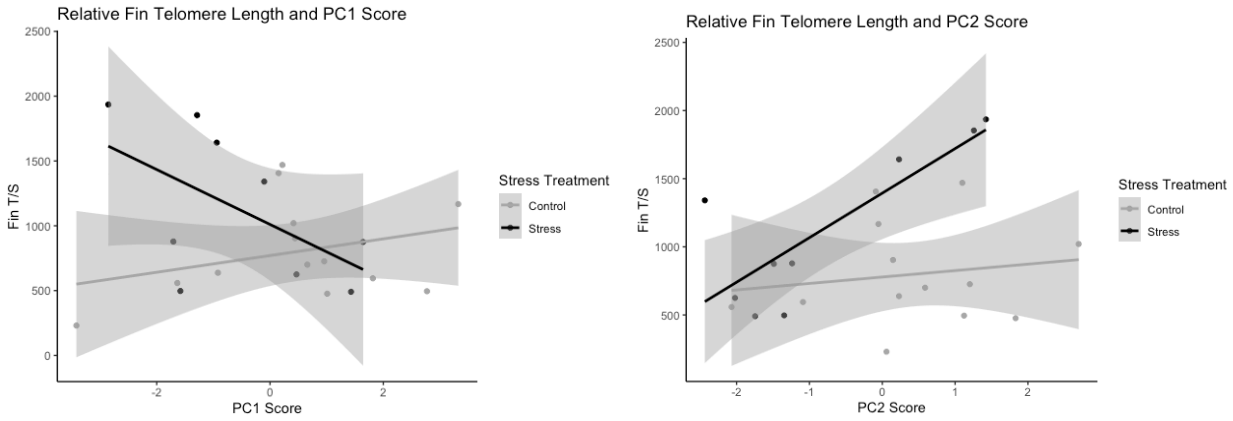

Figure 4.0. Relative fin telomere length as a function of PC scores separated by treatment group. Higher PC1 scores were indicative of exploration open areas where as PC2 was indicative of increased thigmotaxis and locomotion.

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Chapter 4: Discussion

The aim of this thesis was to examine how telomere dynamics vary with age, tissue type and with stress experience in cichlids. In chapter two I demonstrated that telomere length in *A. Burtoni* fin increases with age while liver telomere length remains stable. Sex may be an important factor in telomere length differences. Increased fecundity may be prioritized early in life for female fish causing shorter telomeres internally and after reproduction diverts energy to predator avoidance and fin maintenance. Reproductive output and telomere maintenance have some evidence to suggest they are part of a trade-off strategy where more fecund fish have reduced life expectancy and shorter telomeres (Gao, Munch, 2015). Age at maturation, sex and rearing temperature have also been shown to effect telomere length in sticklebacks suggesting that females may be devoting more resources to reproduction and may trade off this investment with a shorter life expectancy (Noreikiene, Kuparinen, & Merilä, 2017). In chapter three I demonstrated that behavioural differences can been seen in fish that have been exposed to early-life stress. Fish that were exposed to early life stress adopted a more reactive coping style after stress exposure.

My initial hypothesis was that there would be some change in telomere length as fish age, both in liver and fin tissue. Most evidence suggests that telomere length in ectotherms shortens or remains the same as the organism ages (Gao & Munch 2015; Gopalakrishnan et al., 2013; Näslund et al., 2015) however, some evidence has shown that telomere length can increase with age (Tsui, 2005) or early in development (McLennan et al., 2018). This was supported in fin tissue as we saw an increase in relative telomere length as fish got older. However, it is important to note that fish in chapter 2 were in communal tanks where fin damage could occur due to an upregulation of telomerase from fin regeneration whereas in chapter three fish were housed separately. In chapter 3 my hypothesis was that telomere length would decrease with

stress exposure. Here, I found no main effect of treatment of telomere length but these fish were housed together with clear barriers so no fin damage could occur, we therefore know that any increase in telomere length is not due to fin regeneration. There was an interaction with fin telomere, stress treatment and change in exploration activity indicating some relationship. The higher relative fin telomere length in individuals who explored less individuals and who experienced stress could indicate that the stress response is allocating more resources to maintaining and conserving locomotor abilities as they learn that the environment is unpredictable. Cichlids are not always prey as adults they are often predators unless specific apex predators are present, this could suggest that their antipredator responses are heterogenous across time (Menuthen et al., 2019). The frequency of the stressor could signal to the fish that their energy demands can be met during a safer period signalling to the experimental group to not explore. Early life experiences when the fish are more vulnerable could give information to the fish about the environment and program how the respond later in life.

In humans, telomere disfunction can have extremely detrimental effects. Individuals born with abnormally short telomeres or genetic abnormalities that impair telomerase functioning such as dyskeratosis congenita or progeroid syndromes have a range of issues that cause accelerated ageing and premature death (Caldo & Young 2012; Carrero, Soria-Valles, & López-Otín, 2016). Aplastic anemia has much similar characteristics but is instead from an environmental exposure that results in similar consequences through genetic damage (Caldo & Young 2012). For humans, telomeres play an important role in maintaining chromosomal stability while also being and important factor in maintaining the balance between cancer risk and cellular senescence (Monaghan, & Haussmann, 2006). Humans and birds tend to show a relationship between age and telomere length; however, fish do not seem to fit this pattern.

While many fish species do show a negative relationship with telomere length and age (Harmann et al., 2009; Rollings et al., 2014; Hatakeyama et al., 2008; Gopalakrishnan et al., 2013) many show no relationship at all (Gao & Munch 2015; Lund et al., 2009; Näslund et al., 2015; Izzo 2010). Telomeres are highly conserved across eukaryotes so it seems improbable they would not provide some benefit to an organism or play some role in fitness considering the wide implications they have in humans. While telomere length may not be a useful indicator of absolute age in fish it may be a useful bioindicator (Chatelain, Drobniak, & Szulkin, 2020). In humans, the telomere position effect has also been shown to make reversible epigenetic changes to nearby genes and thereby affecting cellular senesces mechanisms and telomerase regulation (Baur et al., 2001; Shay, 2018). Fish show higher levels of telomerase expression throughout their lives than humans possibly due to their ability to grow indeterminately (Hatakeyama, et al., 2018) and may retain the ability to lengthen telomeres into adulthood (Mclennan et al., 2018; Peterson, Mok, & Au, 2015). Organisms that lengthen their telomeres could also be of particular importance in examining alternative telomere lengthening. Alternative telomere lengthening is responsible for telomere lengthening by using itself or other telomeric DNA as a template without telomerase and is thought to be a cause for continuous growth in 10% of human tumors (Henson, Neumann, Yeager, & Reddel, 2002). With many model species absolute telomere length varies widely so increasing evidence suggests the rate of loss and the lifespan may be the most important indicator for model species (Barrett et al., 2012; Wilbourne et al., 2018). Our results do not support this however there is evidence that a longer time span could reveal a relationship similar to what has been found in the literature (Chatelain, Drobniak, & Szulkin, 2020; Mclennen et al., 2018).

The current study allows for the examination of these dynamics in fish that are genetically similar to one another. There is some evidence however, that lab bred populations show less age-related telomere effects than those in the wild (Izzo, Bertozzi, Gillanders & Donnellan 2014). Wild population lines seem to show more diverse behavioural characteristics (Koolhaas et al., 2011). The current study does lack some of the ecological validity that using wild population would allow for, but taking into account the high heritability estimates of telomere length (Asghar et al. 2015) examining a population with less intraspecies variation is much more valuable for initial demonstrations of this relationship. Lab bred animals may show survival bias where more aggressive and bold animals fight more frequently and may get injured or die as a result leaving only the shyer animals. Fish who are brought into the lab may also be more homogenous due to capture order effects in regard to size and behaviour (Härkönen, et al., 2016; Vainikka, et al., 2012). Wild populations of birds also indicate that coping style depends on the stability of the environment and social structure (Koolhaas et al., 2011); introducing more instability in social structure or food availability the lab environment may result in more pronounced effects. However, introducing instability does create more confounds in the relationship and possibility of mortality. Strong maternal influence has also been observed in relation to early life telomere length in reed warblers (Asghar et al. 2015) suggesting that having parental data would be especially beneficial in determining how telomere length is inherited and how it may change in relation to stress.

Pre- and post-exposure sampling would allow for further detail on the time necessary to impact telomere length. Studies have shown that growth rate can be quite plastic and change rapidly (Hofmann, Benson, & Fernald, 1999). Sampling individuals on multiple occasions can determine how quickly permanent damage can occur if compensatory growth takes place. The

rate in which telomere length changes could be more informative than length at a given time point (Sudyaka et al., 2016). Longitudinal studies in bird species have taken multiple samples of the course of the lifetime in wild Seychelles warbler (*Acrocephalus sechellensis*) and found that telomere length decreased with age and was a better predictor of mortality than age (Barrett et al., 2013). In fish there is fewer studies who have examined telomere length in the same animal across time. Wild Brown trout (*Salmo trutta)* sampled for fin tissue and then muscle tissue samples 3-4 month later were found to have no relationship in regard to telomere length (Adriaenssens, Pauliny, Blomqvist, & Johnsson, 2016), however, previous studies indicate that there is repeatable inter-individual variation within tissue types in brown trout (Näslund et al., 2015). Additionally, Naslund et al., (2015) found that there were no treatment effects of food deprivation on brown trout after one year but found a negative correlation between initial size and the subsequent change in telomere length suggesting they be able to maintain telomere length but delayed cost.

The level and timing of the stressor is important for determining the severity of the stressor for an individual, as can be seen in studies of predictability of stress (Galhardo, Vital, & Oliveira, 2011). It is possible that moderate net chasing stress is not sufficient to produce these kinds of effects in telomere length during the given time period. Predatory stress has shown some induced neophobia (Elvidge, Chuard, Pierre & Brown, 2016) however, the amount of stress required to make permanent changes on the individual level may need to be either more intense or more chronic in nature (Clinchy, Sheriff and Zanette, 2013; Archard, Earley, Hanninen, & Braithwaite, 2012). High predation groups of poeciliid fish, *Brachyrhaphis episcopi* showed more exploratory behaviour than those in the low predation group who exhibited more neophobic behaviour much like the stress treatment group in chapter 3 (Archard, Earley,

Hanninen, & Braithwaite, 2012). Similar results were found in Panamanian bishop (*Brachyrhaphis episcopi)* where fish in a high predation group showed more exploratory and proactive coping than those in low predation groups (Archard, & Braithwaite, 2011). However, in these studies fish in the high predation groups were wild caught from rivers where high amounts of predatory fish are found, one of which being cichlids (Archard, & Braithwaite, 2011). Other methods including heat stress and low of food availability have been shown to induce oxidative stress (Kim, Noguera & Velando, 2019) and telomere length attrition in other fish species (Simide et al., 2016). Exposure to the pesticide chlordecone in cichlid fish was also shown to stimulate oxidative stress in liver, gill and brain tissue (Asifa, & Chitra, 2017). Further studies may be needed to examine differing timing and severities of stressors to assess the continued impact of social and environmental stressors. Measurements of longevity and fitness would also be beneficial for gaining a broader picture of how telomere dynamics affect the life course of cichlids.

My aim for this thesis was to determine how telomere length varies in cichlids with age and with stress exposure. Telomere length in *A. Burtoni* was shown to increase with age in fin tissue but not in liver tissue. Telomere length in A. nigrofaciata did not vary with early life stress but did interact with behaviour and fin. From this we can gain reasonably assume that fin is a better indicator of telomere length than liver tissue.

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