TELOMERE LENGTH AND SENESCENCE IN A LONG-LIVED FISH (*ICTIOBUS CYPRINELLUS*)

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ABSTRACT

The pattern of shorter telomere lengths in older individuals has been observed across many vertebrates, but has not been well-documented in bony fishes. Bony fish (i.e., Class Osteichthyes) represent the most speciose and oldest group of vertebrates, and understanding their telomere dynamics can fill gaps in our understanding of the process and evolution of aging in vertebrates. In this study we quantified telomere length, immune function, and stress in bigmouth buffalo *(Ictiobus cyrpinellus*), a long-lived bony fish in the family Catostomidae, to characterize variation in telomeres and condition. We found that length of telomeres extracted from red blood cells was not related to age. We also found that the neutrophil/lymphocyte ratio was lower in older individuals, and immune function was greater in older individuals. Our findings suggest bigmouth buffalo may be capable of intrinsic regulation of telomere shortening and provide support for the existence of negligible senescence in some vertebrates.

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TELOMERE LENGTH AND SENESCENCE IN A LONG-LIVED FISH (*ICTIOBUS CYPRINELLUS*)

Introduction

Telomeres and their association with senescence are a relatively new approach to studying growth, aging, and longevity. Telomeres are repetitive sequences of DNA located on the ends of linear chromosomes which play a protective role in cellular division. Telomere lengths generally get shorter each time a cell replicates, placing a “mitotic clock” on every cell that limits the number of divisions the cell can undergo before its telomeres reach a critically short length. This critical telomere length reduces the ability of cells to function and replicate, which is why telomere length has been associated with senescence and mortality (Horn *et al.* 2010). Senescence results from an accumulation of damage due to the decline in an organism's ability to repair cellular damage, and leads to dysfunction in organism-level processes and ultimately mortality. Critically short telomeres have been shown to be directly associated with DNA damage response proteins (Fagagna *et al.* 2003). However, telomerase is an enzyme that has been shown to add repetitive sequences onto telomeres (Aubert and Lansdorp 2008), potentially buffering cellular damage associated with aging cells. The relationship between telomere length and telomerase is complex, and little is known about this relationship across different species. Still, chronologic age is negatively correlated with telomere length in many vertebrates, including humans.

Telomere length has been negatively correlated with age in humans (Tsuji *et al.* 2002), birds (Haussmann & Vleck 2002), dogs, cattle, mice, primates (Haussmann *et al.* 2003), snakes (Bronikowski 2008), alligators (Scott *et al.* 2006), and sea turtles (Hatase *et al.* 2008). Due to the consistent observation of shorter telomeres in older individuals across species, it is thought that

telomere length can predict lifespan. In fact, Heidinger *et al.* (2011) found that telomere length in the early stages of life was a strong predictor of realized lifespan in zebra finches (*Taeniopygia guttata*).

The connection between age and telomere length has led to the application of telomere length for estimating age and fitness in an ecological context (Horn *et al.* 2010). If age-related changes in physiological processes are a result of telomere length, selection can act on telomere length. Because age and telomere length are correlated, age-related observations related to condition and fitness may be attributable to telomere length. For example, function of the immune system in humans and other mammals degrades with age (Utsuyama et. al 1991). The impacts of this decline in immune function are significant. Roberts-Thomson *et al.* (1974) found that older (age 60+ years) humans with decreased immune function displayed higher rates of mortality when compared to humans with immune function in a normal range. Greeley *et al*. (2001) reported age-related decline in absolute numbers of immune cells such as lymphocytes, T-cells, CD4-cells, and CD8-cells in Labrador retrievers. In a wild population of collared flycatchers (*Ficedula albicollis*), the humoral response of the immune system was significantly weaker in older birds (Chichon *et al.* 2003). The primary hypothesis proposed to explain these patterns is that there is a decrease in helper T-cell activity as an individual grows older. T-cells are made in the thymus during the newborn stages of life, but very few cells are made during adulthood (Makinodan & Kay 1980). In mice, the ability of the thymus to create T-cells changes with age, as does the characteristics of the T-cells created (Utsuyama *et. al* 1991).

The age-related changes observed in immune function may be a result of changes in telomere length. The immune system is thought to be sensitive to the shortening of telomeres because immune system function relies on cell renewal and replication of T and B-cells

(Kaszubowska 2008). Telomere shortening limits the proliferation of these cells, and consequently limits the capacity of the immune system. Damjanovic *et al.* (2007) suggest that telomere loss negatively alters T-cell function and accelerates the aging of T-cells in humans, resulting in a decline in immune function. A number of immune-related diseases have been associated with shorter telomere length (Andrews *et al.* 2010). In horses, decreased telomere length correlated with decreased proliferative response in peripheral blood mononuclear cells (Katepalli *et al.* 2008).

In fishes, the relationship between age and telomere length is uncertain. Telomere dynamics follow a consistent pattern among terrestrial vertebrates; in general, telomere length shortens across the lifespan, causing changes in cellular function. However, lifespan, the source of telomeric DNA, sex, environmental conditions, and telomerase levels can alter the dynamic relationship between age and telomere length in fishes. In laboratory studies, Eastern mosquitofish (*Gambusia holbrooki*) displayed telomere shortening with increasing age, but water temperature also affected telomere length (Rollings *et al.* 2014). Anchelin *et al.* (2011) found that telomere length in zebrafish (*Danio rerio*) does not steadily decline with age, but sharply decreases at old ages (despite the expression of telomerase at all ages). Wild-strain turquoise killifish (*Nothobranchius furzeri*) exhibit age-dependent telomere shortening despite high telomerase activity, but their laboratory-strain counterparts do not (Hartmann *et al.* 2009). Common carp (*Cyprinus carpio*) show a positive correlation between body size (which is positively, but non-linearly correlated with age) and telomere length (Izzo *et al.* 2014), while Japanese medaka (*Oryzias latipes*) display differences in telomere lengths between male and female fish (Gopalakrishnan *et al.* 2013). Furthermore, Hatakeyama *et al.* (2016) found that telomere length does not steadily decline in Japanese medaka, but is a reflection of the variation

in telomerase activity throughout different life stages. The uncertainty surrounding telomere dynamics in fishes is substantial, and creates a gap in our understanding of aging and telomere length in vertebrates.

Some extrinsic factors have been shown to influence the rate of change in telomere length. For instance, chronic stress has been shown to accelerate telomere loss (Kotrschal *et al.* 2011). In fact, von Zglinicki (2002) suggests that stress-induced DNA damage may cause more telomere erosion than cellular replication. Shorter telomere length and decreased telomerase activity was observed in mothers of chronically ill children when compared to mothers of healthy children (Epel *et al.* 2004). Moreover, mothers that were caretakers for more years showed decreased telomere length compared to mothers that were caretakers for shorter time periods (Epel et al. 2004). Tyrka *et al.* (2010) found that adults exposed to abuse during childhood had shorter telomeres than adults of the same age that were not exposed to abuse. In king penguins (*Aptenodytes patagonicus*) high levels of oxidative stress are correlated with shorter telomere length in DNA from red blood cells (Geiger *et al.* 2012). Chronic environmental stress appears to affect telomere length and the rate of cellular aging, suggesting that stress response plays an important role in telomere dynamics.

White blood cell enumeration provides a quantitative measure of stress in vertebrates. The effect that glucocorticoids have on white blood cell production is consistent across vertebrate taxa. Specifically, an increase in glucocorticoid hormones will increase the relative number of neutrophils and decrease the relative number of lymphocytes in the blood (Davis *et al.* 2008). Therefore, an increase in glucocorticoid concentrations leads to an increase in the neutrophil to lymphocyte ratio (henceforth, NLR) within an individual. Chronic exposure to stress, and subsequent chronic elevation in glucocorticoid concentrations can be quantified

among individuals using the NLR (Vleck *et al.* 2000) Neutrophils and lymphocytes can be identified in fish blood smears, and the observed patterns appear identical to those seen in other vertebrates (Davis *et al.*, 2008). Furthermore, there is time lag between the exposure to a stressor, change in glucocorticoid concentration and the expression of a leukocyte response. This time lag is longer in ectothermic organisms (Davis *et al.* 2008), possibly due to their relatively slow metabolism compared to endotherms. The effects of handling and transport stress were not observed in the white blood cell counts in channel catfish (*Ictalurus punctatus*) for 12 hours, and the maximum response was observed only after 24 hours (Bly *et al.* 1990). In practice this time lag allows for the quantification of stress levels experienced prior to handling, capture or transport in free-living individuals.

Fish can be exposed to a variety of environmental stressors in their natural habitat, including those caused from anthropogenic substances (Iwama 1998). In prior research, fish have been exposed to a variety of stressors including cold water temperatures (Bennett & Gaudio Neville 1975), constant daylight (Valenzuela, Silva, & Klempau 2008), and handling and translocation (Ellsaesser & Clem 1986), all of which caused an increase in NLR. In fact, researchers have even utilized NLR to quantify the effects of heavy metals on a variety of fish species (Witeska 2005, Dethloff *et al.*, 1999, Dick and Dixon 1985).

The ratio of neutrophils to lymphocytes (henceforth, NLR) can also give insight into the life stage of individuals. In healthy human populations, older individuals have higher NLR than younger individuals (Li *et al.* 2015), and an increase in NLR with age has also been observed in horses (Satue *et al.* 2009). Aging individuals tend to exhibit overall deteriorating condition, suggesting the NLR may be correlated with condition as well. In fact, in birds low lymphocyte counts have been associated with increased susceptibility to disease (Al-Murrani *et al.* 2002),

slow growth rates (Moreno *et al.* 2002), and lower survival to the next breeding season (Lobato *et al.* 2005), indicating that leukocyte quantification can provide information about the fitness of individuals.

Quantifying the relationships among age, stress, immune function, and telomere length can give us information about the onset and progression of senescence. Finch (1994) categorized senescence into three categories based on rapid, gradual, or negligible rates of progression. Fish appear to display great diversity in their senescence patterns, but some long-lived fishes that grow indeterminately show minimal signs of senescence (Finch 1998). Fishes exhibit asymptotic, but indeterminate growth instead of the terminal growth seen in mammals and birds. Indeterminate growth in fish suggests that all somatic cells require a high replication capacity. Klapper *et al.* (1998) investigated telomerase activity in different tissues of rainbow trout (*Oncorhynchus mykiss*) and found relatively high levels of telomerase activity in liver, skin, heart, muscle and brain tissue regardless of age and size. Brain and muscle tissue showed the lowest levels of telomerase, but even these levels were similar to fast growing tumor cells in humans (Klapper *et al*. 1998). In humans, telomerase is only expressed in tissues with high proliferation capacity, such as stem cells (Greider 1998). The telomere dynamics of long-lived fishes with lifespans similar to that of humans are not known, but could provide insight into differences in the aging process in indeterminate versus determinate growth.

Bigmouth buffalo (*Ictiobus cyprinellus*) are a long-lived, freshwater fish in the Family Catostomidae. Bigmouth buffalo are native to the Mississippi River basin from Canada to the Gulf of Mexico (Johnson 1963), inhabiting shallow lakes and slow-moving rivers where they feed on zooplankton suspended in the water column (Adamek *et al.* 2003). Until recently, bigmouth buffalo life history (e.g., growth rate, lifespan, reproductive maturation) had not been

accurately quantified. Recent research using otoliths indicates bigmouth buffalo from the Red River of the North (henceforth, Red River) and Mississippi River basins in Minnesota can live to 110 years (Lackmann *et al.* in review). Their growth rate is asymptotic, increasingly slowly after age 20 (Lackmann *et al.* in review). Females reach larger sizes than males and may not spawn every year (Johnson 1963). Sexual maturity is reached at 7-10 years of age (Lackmann *et al.* in review).

These life history traits make the bigmouth buffalo an ideal fish species for studying telomere dynamics and aging. The majority of studies on telomere length in fish have focused on short-lived species. The lifespan and growth rate of bigmouth buffalo is comparable to that of humans, yet their poikilothermic, indeterminate growth reflects differences indicative of bony fishes. In addition, teleost fishes comprise about half of all vertebrate species (Finch 1994), making them a crucial part of understanding vertebrate evolution and life history. We measured telomere length in DNA from red blood cells, muscle tissue, and gonadal tissue in bigmouth buffalo spanning a wide range in age. We also quantified chronic stress using NLR and measured immune function of individuals using a bactericidal assay. Using these measurements, we looked for correlations among and between age, telomere length, stress, and immune function to gain understanding of telomere dynamics and the progression of senescence in long-lived fish.

Methods

Fish Collection

Bigmouth buffalo were collected in Minnesota from sites within the Red River and Mississippi River basins. Fish were obtained from Artichoke Lake (date of collection May 4th, 2017) and Lake Minnetaga (Sept 22nd, 2017) in the Mississippi River basin and from the Ottertail River (just downstream of Orwell Dam) (April 7th, 2018), Lake Lizzie (August 2017), Pelican

Lake (August 2016 through July 2017), Rush Lake (May 2018), and North Lida Lake (July 2017) in the Red River basin. Fish from Artichoke and Minnetaga Lakes were purchased from commercial fisherman, but all of the other fish were obtained from recreational fisherman. Immediately following collection, total length (± 0.1 cm) and wet mass (± 0.1 kg) were recorded from each fish.

A small blood sample was obtained from as many individual fish as possible. Following collection and measurements of size, fish were euthanized by overanesthetizing with tricaine methanesulfonate and a blood sample (approximately 3 mL) was obtained from a gill arch and placed in a heparinized container (BD Vacutainer®). Following blood collection, whole fish carcasses were placed on ice and returned to the lab at North Dakota State University within three hours where they were frozen at -20 C. Blood samples were also placed on ice and returned to the lab, where a small drop of whole blood was smeared on a glass microscope slide for each sample, then the samples were centrifuged (1700 G for 10 minutes) for plasma separation. Individual plasma samples were removed with a pipette and placed in 1.5 mL Eppendorf tubes, and the packed red blood cells were left in the original BD Vacutainer®, then red blood cell and plasma samples were frozen (-20º C) until later analysis. Blood smears were allowed to air dry, then stained with a Hemacolor® staining kit.

Dissection

Carcasses were dissected to determine sex, collect tissue samples for DNA extraction, and collect otoliths for age determination. Muscle biopsies were obtained by cutting approximately 25 grams of muscle tissue from the ventral side of each fish. Whole gonads were extracted and weighed (± 0.1 g) from each fish, and sex recorded for each individual. The tissue samples were then frozen (-20º C) until use in DNA extraction. As many otoliths as possible

were extracted from each fish by cutting into the cranium and first vertebral column from the ventral side of the fish and removing the labyrinth organ. Otoliths were extracted from the labyrinth organ and stored at room temperature in 1.5 mL Eppendorf tubes filled with water prior to preparation for age analysis.

We obtained age estimates from counts of annuli in thin-sectioned otoliths, as explained in Lackmann *et al.* (in review). We will briefly describe the aging procedure here. Otoliths were removed from the fish, rinsed clean and dried for 30 minutes at 55°C then embedded in ACE® quick-setting epoxy within 1.5 cm^3 compartments (lined with petroleum jelly) in a plastic tray. After the epoxy hardened, the epoxy block was placed in a Buehler IsoMet™ 1000 low-speed saw equipped with diamond-embedded thin-sectioning blades to obtain 300-500 µm sections via the wafer method (Campana *et al.* 2008). Thin sections of the otoliths were mounted on a glass microscope slide, photographed at 75X under a compound microscope using transmitted light, and images were then examined for annuli that could be quantified and were digitally marked. Otolith sections were assigned ages by multiple readers, with consensus readings used to determine the final age assigned to each specimen. First, a primary and secondary reader independently marked annuli on duplicate images of the thin section. Discrepant annuli counts between the primary and secondary reader were identified using a minimum criterion of one year per decade of age. For example, reader counts for individuals scored 0-9 years of age were deemed discrepant if the primary and secondary reader scores differed by more than ± 1 annulus count. This approach was used for individuals scored up to 110-119 years (deemed discrepant if the primary and secondary reader scores differed by more than ± 12 annulus counts). Images of otoliths identified as discrepant were then either independently analyzed by a third reader (n=29), or another otolith section(s) already available from the same fish was aged by both primary and

secondary readers. If consensus scores were still not obtained between readers, then yet another otolith was thin-sectioned from that specimen and again scored independently by the primary and secondary reader, at which point all age estimates were resolved. Otoliths for which annuli counts were not identical between readers, but not identified as discrepant (*e.g*., scored 12 by the primary and 13 by the secondary), a final age determination was made by the primary reader. The overall between-reader precision (primary and secondary) had a coefficient of variation (CV) of 5.6%. Precision varied with age and was highest in the youngest group of fish. For individuals across each of the 12 decadal age groups in this study (from 0-9, to 110-119 years) the CV was 10.4, 5.7, 4.0, 4.5, 4.5, 3.6, NA, 3.3, 2.9, 3.4, and 2.7, and 3.9% respectively (Lackmann *et al*., in review).

Leukocyte Analysis

We obtained counts of leukocytes from the stained blood smears. Smears were examined under a compound microscope (400X magnification), and neutrophils and lymphocytes were counted until the combined total count exceeded 100 (Vleck *et al.*, 2000). NLR was calculated by dividing the number of neutrophils by the number of lymphocytes. We also recorded the number of microscope viewing fields required to achieve the 100 cell count for the NLR.

Telomere Length Analysis

We determined telomere length from DNA extracted from red blood cells, muscle tissue, and gonadal tissue. Genomic DNA was extracted from blood using a Nucleospin® Blood kit (Macherey-Nagel, Inc.) and from muscle and gonadal tissues using a Nucleospin® Tissue kit (Macherey-Nagel, Inc.). Concentration and purity of extracted DNA was measured using a NanoDrop™ spectrophotometer. DNA extractions were then diluted with ultra-pure water to approximately 200 ng/µl, mixed with Promega Blue/Orange 6X Loading Dye (Promega, Inc.),

and loaded into ethidium-bromide enriched agarose gel wells. Gel electrophoresis was conducted for approximately 1.5 hours at 30 V before the gel was illuminated with UV light and photographs were taken. Only DNA extractions with full integrity were utilized in telomere length analysis.

Telomere length was measured using quantitative PCR (qPCR) on an Mx3000P qPCR system (Stratagene, Cheshire, UK). This method yields a relative measure that can be used for comparison within and among individuals of the same species (Heidinger *et al.* 2016). We followed the methods of Heidinger *et al.* (2016) with slight modifications for species. Modifications included using beta-actin primers (from the available genome of a species related to bigmouth buffalo) as the control, single copy gene, and measuring each reaction in duplicate. The suitability of our control, single copy gene was tested by a melt curve analysis, which established that the dissociation curve had a single peak. Telomere and beta-actin reactions were undergone on separate plates. Telomere length was calculated as the ratio (T/S) of telomere repeat copy number (T) to control, single gene copy number (S) of the focal sample relative to a reference sample (Heidinger *et al*. 2016). Telomere length from blood was calculated across three microplates, while telomere length from gonad and muscle each had their own single plate. This eliminated inter-plate variation for gonad and muscle telomere measurements. Intra-plate variation in cycle threshold (Ct) values among duplicates was calculated for all plates. An arbitrary, single sample was run on all red blood cell plates to allow for the calculation of inter-plate variation of T/S ratios that were run on three separate plates. Every plate also included a blood sample from the same single individual that was serially diluted to produce a standard curve, and used to measure reaction efficiencies.

Immune Function Analysis

We quantified immune system strength using a bactericidal assay. This assay assesses the ability of the immune system to eliminate an actual pathogen and involves the use of phagocytes, opsonizing proteins, and natural antibodies (French *et al.* 2010). We used a bactericidal assay described by Zysling *et al.* (2009) with the following modifications: we used plasma instead of whole serum, measured each sample in triplicate, and adjusted concentrations to yield control plates with approximately 250 bacterial colonies. Briefly, a working solution of *E. coli* (EpowerTM Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN) was mixed with serum that was diluted with CO_2-independent media. This solution was activated by incubation at 37º C for 30 minutes. Fifty microliters (50 µl) of solution was then plated on tryptic soy agar plates and allowed to incubate overnight. Control plates were created by diluting the working solution with media alone. Colonies were counted on each plate and the mean number of colonies on sample plates was divided by the mean number of colonies on control plates. This fraction was subtracted from 1 and multiplied by 100 to express killing capacity as the percentage of bacteria killed relative to control plates.

Statistical Analysis

We analyzed relationships among age, body size, condition, sex, site, telomere length, immune system strength, and NLRs using general linear models. Telomere lengths and NLRs followed a log-normal distribution, so we log-transformed both for statistical analysis. Killing capacity was logit-transformed. We obtained residuals (on the mass axis) from an orthogonal regression of log-transformed mass and log-transformed total length of males and females separately, and refer to the residual as condition. We analyzed relationships among blood telomere length, gonad telomere length, muscle telomere length, NLR, and killing capacity using

Pearson's correlation coefficient. We used paired t-tests to compare telomere lengths from different tissues within the same individual. We used ANCOVA to look for effects between killing capacity, NRL, age, and site. We assumed statistical significance at $\alpha=0.05$. All statistical analyses were conducted using JMP 13 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Age and Size Distributions

We obtained size, age and sex from 240 individuals. Ages of fish ranged from 2-102 years old and were not uniformly distributed among sites (Figure 1). Individuals from Lake Minnetaga were significantly younger than individuals from Rush Lake, Lake Lizzie, North Lida Lake, and Pelican Lake (Figure 1). Individuals from Orwell Dam and Artichoke Lake had similar age distributions (Figure 1). Total lengths ranged from 30.7-96.9 cm and masses ranged from 0.45-14.33 kg, with females reaching larger size at age than males (Figure 2). Like age, size was not uniformly distributed among sites. An orthogonal regression indicated log-transformed total length was significantly correlated to log-transformed mass (for females: ln total length = 1.35 + 0.34 * ln mass, $R^2 = 0.99$, n = 128; for males: ln total length = 1.37 + 0.34 * ln mass, $R^2 = 0.98$, n = 127). Residuals from the regression were used to quantify condition.

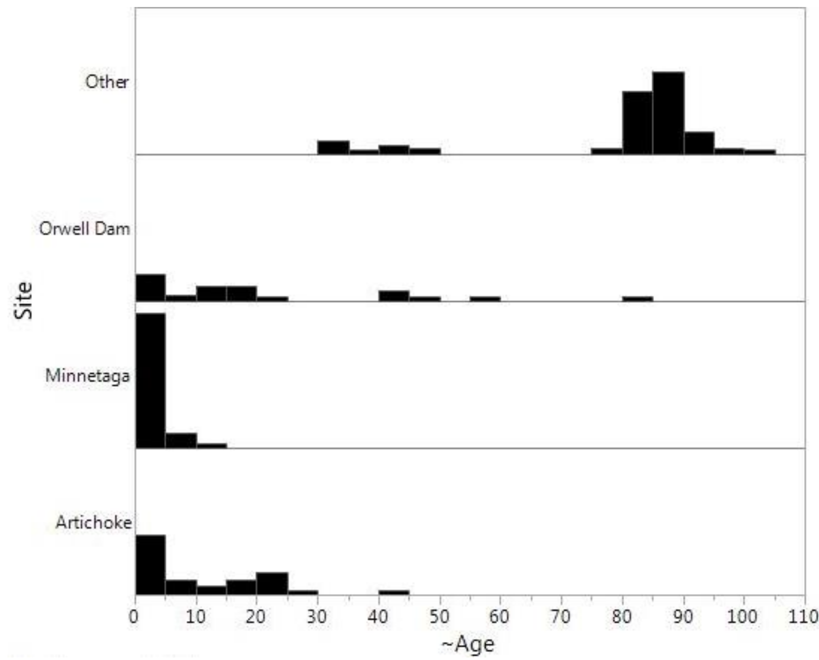

Figure 1. Age distributions of bigmouth buffalo collected from different sites throughout Minnesota.

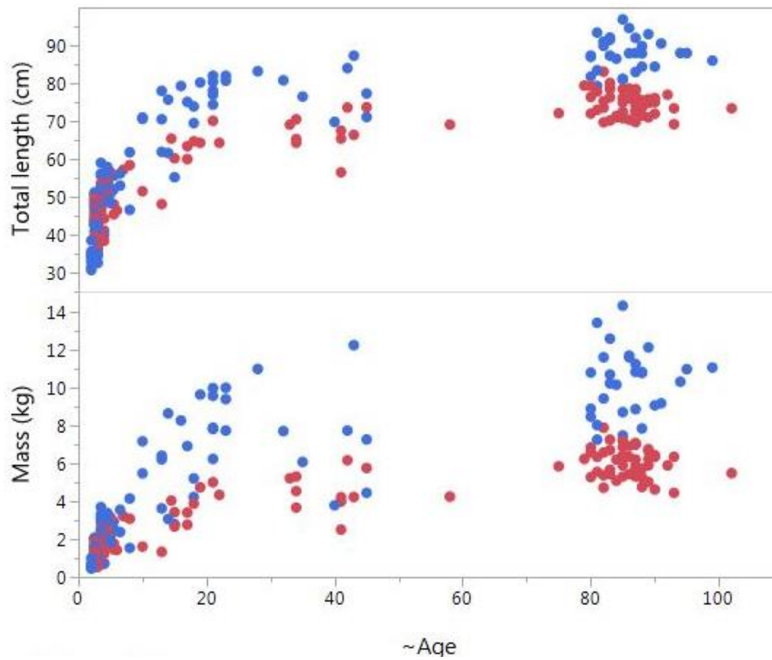

Figure 2. Age and size (total length above, mass below) for individual bigmouth buffalo. Red dots represent males and blue dots represent females.

Neutrophil/Lymphocyte Ratio

We were able to quantify NLR from 92 individuals. These 92 individuals came from Artichoke Lake (37), Lake Minnetaga (24), Rush Lake (9) and the Ottertail River below Orwell Dam (21). Age explained approximately 48% of the variation in log-transformed NLR ($F_{1,88}$ = 78.26, p < 0.01, r^2 = 0.48) (Figure 3). Total length ($F_{1,91}$ = 27.3, p < 0.01 r^2 = 0.23), mass ($F_{1,91}$ = 27.4, p < 0.01, r^2 = 0.23) and condition ($F_{1,92}$ = 8.54, p < 0.01, r^2 = 0.09) also explained a significant amount of NLR variation, but sex did not explain any of the variation ($F_{1,92}$ = 0.00 p = 0.95 r^2 < 0.01) in NLR. Although site explained 46% of the variation in NLR ($F_{3,91}$ = 24.71, p < 0.01, r^2 = 0.45), this was due to skewed age distributions at certain populations. We ran a restricted ANCOVA with only individuals from Artichoke Lake and the Ottertail River below Orwell Dam because these populations had similar age distributions with range similar to that of the overall sample. The restricted ANCOVA showed age ($F_{1,59}$ = 7.82, p < 0.01, r^2 = 0.12) and the age by site interaction ($F_{1,59}$ = 6.24, p = 0.02, r^2 = 0.08) had a significant effect on NLR, but site alone did not ($F_{1,59}$ = 0.49, p = 0.49, r^2 = 0.01). In addition there was a significant increase in the number of microscope fields required to count at least 100 leukocytes (necessary for determining the NLR) with age at Artichoke Lake ($F_{1,37}$ = 7.29, p = 0.01, r^2 = 0.17), and an increase that approached significance for all sites combined ($F_{1,88}$ = 3.78, p = 0.06, r^2 = 0.04), but not for individuals from the Ottertail River below Orwell Dam ($F_{1,22}$ = 2.99, p = 0.09, r^2 = 0.13). NLR was negatively correlated with telomere length from blood (R = -0.27, n = 63, p = 0.03), but not significantly correlated with telomere length from muscle (R = 0.18, n = 26, p = 0.37) or gonad (R = -0.31, n = 18, p = 0.21).

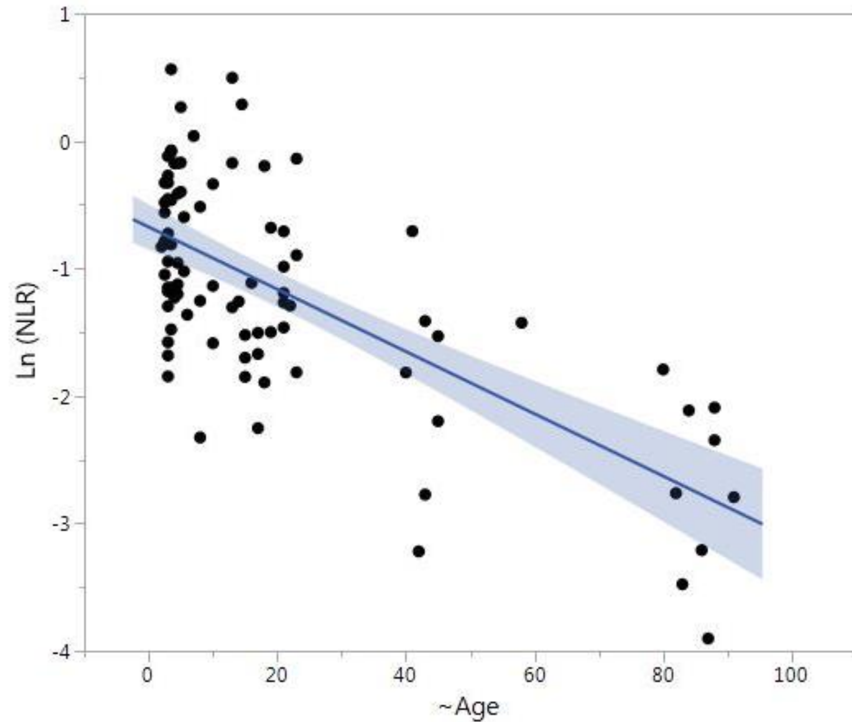

Figure 3. Age and log-transformed NLR for bigmouth buffalo individuals.

Telomere Length

Telomere length was successfully quantified from genomic DNA in red blood cells (97 individuals), muscle (29), and gonad (26) of bigmouth buffalo. The average reaction efficiencies for the beta-actin (mean ± 1 SEM: 94.8 ± 1.22%) and telomere (101.1 ± 0.62%) plates were close to 100%, so we did not adjust for efficiency differences when calculating telomere lengths. For red blood cell telomere measurements, the inter-plate coefficient of variation for the repeated sample T/S ratios was 11.0%. Reactions were highly replicable, with the average coefficient of variation between replicates across all plates equal to 0.52%. Age did not explain any of the variation in telomere length from blood (($F_{1,97} = 0.65$, $p = 0.42$, $r^2 = 0.01$) (Figure 4), muscle ($F_{1,29} = 0.12$, $p = 0.73$, $r^2 < 0.01$), or gonad ($F_{1,26} = 2.55$, $p = 0.12$, $r^2 = 0.09$). Total length and mass, respectively, did not explain a significant amount of the variation in telomere length from blood ($F_{1,97} = 0.72$, $p = 0.40$, $r^2 = 0.01$) ($F_{1,97} = 1.58$, $p = 0.21$, $r^2 = 0.02$) , muscle ($F_{1,29} = 0.76$, p

= 0.39, $r^2 = 0.03$) ($F_{1,29} = 0.58$, $p = 0.45$, $r^2 = 0.02$), or gonad ($F_{1,26} = 0.39$, $p = 0.54$, $r^2 = 0.02$) ($F_{1,26} = 0.61$, $p = 0.44$, $r^2 = 0.03$). Sex and condition also failed to explain a significant amount of variation in telomere length from blood ($F_{1,97} = 0.05$, $p = 0.82$, $r^2 = 0.00$) ($F_{1,97} = 1.09$, $p = 0.30$, $r^2 = 0.01$), muscle ($F_{1,29} = 1.64$, $p = 0.21$, $r^2 = 0.05$) ($F_{1,29} = 0.00$, $p = 0.97$, $r^2 < 0.01$), or gonad ($F_{1,26} = 0.02$, $p = 0.66$, $r^2 = 0.01$) ($F_{1,26} = 0.00$, $p = 0.97$, $r^2 = 0.00$). Site did not explain a significant amount of the variation in telomere length from blood ($F_{1,97} = 2.22$, $p = 0.06$, $r^2 = 0.11$). Muscle and gonad telomere lengths were each measured from a single population, so site could not explain any variation in telomere lengths from these tissues. Telomere length from muscle (R = 0.66, n = 26 $p < 0.01$) correlated positively with telomere length from blood, and telomere length from gonad approached a significant positive correlation with telomere length from blood (R = 0.51, n = 14, $p = 0.06$). Mean telomere length from blood (-0.35 ± 0.04) was significantly shorter than gonad (-0.044 ± 0.07) and muscle (-0.08523 ± 0.07) (F = 11.05, $p < 0.01$, $r^2 = 0.13$). A paired t-test indicated that mean blood telomere length (-0.3708) was significantly shorter than gonad (0.00347) (n =14, t = 4.018, $p < 0.01$) for individuals from which we measured telomeres from both tissues. A paired t-test indicated that mean blood telomere length (-0.2938) was also significantly shorter than muscle (-0.085) (n = 26, t = 3.154, $p < 0.01$) for individuals from which we measured telomeres from both tissues.

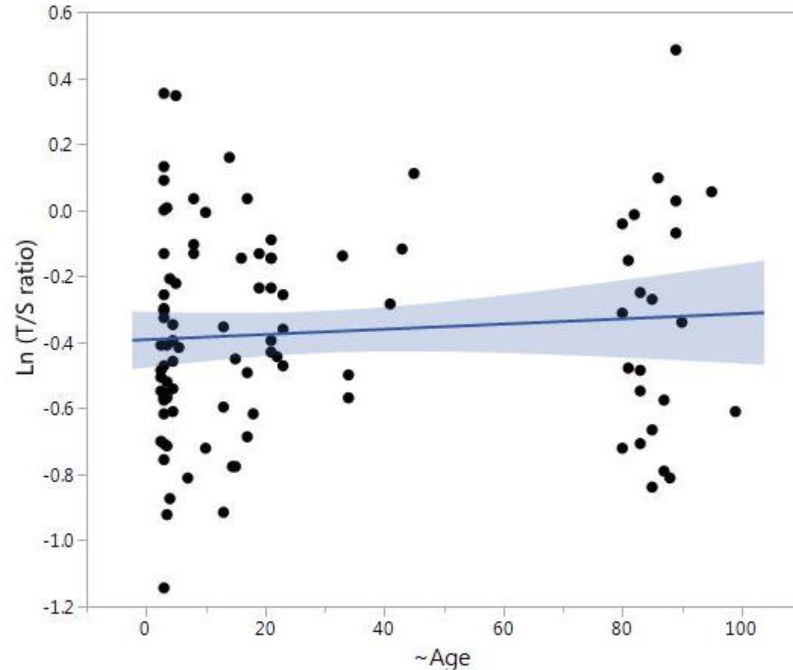

Figure 4. Age and relative telomere length measured from red blood cells (expressed as the natural log of the T/S ratio) of bigmouth buffalo.

Bacteria Killing

Immune function was successfully quantified in 89 individuals. These 89 individuals came from Artichoke Lake (31), Lake Lizzie (12), Lake Minnetaga (25), North Lida Lake (1), Ottertail River below Orwell Dam (16), and Pelican Lake (4). Total length ($F_{1,87} = 1.21$, $p = 0.27$, $r^2 = 0.01$), mass ($F_{1,87} = 0.23$, $p = 0.63$, $r^2 < 0.01$), and condition ($F_{1,89} = 0.01$, $p = 0.91$, $r^2 < 0.01$) did not explain a significant amount of the variation in bacteria-killing capacity, but sex explained approximately 4% of the variation ($F_{1,87} = 3.99$, $p = 0.04$, $r^2 = 0.04$). Site explained a significant amount of the variation in killing capacity ($F_{1,88} = 12.40$, $p < 0.01$, $r^2 = 0.37$) (the single individual collected from North Lida Lake was excluded from this analysis). Individuals from Lake Minnetaga had significantly higher bacteria-killing capacity than fish from the other sites combined ($F_{1,89} = 30.11$, $p < 0.0001$, $r^2 = 0.25$). However, individuals from Minnetaga were all younger than 15 years old. Because of this, we separated Minnetaga individuals from all

individuals from other sites and separated individuals less than 15 years old from individuals greater than 15 years old for comparison. An ANOVA analyzing only individuals under the age of 15 years old showed that individuals from Minnetaga had significantly higher killing capacity than individuals from all other sites combined ($F_{1,51} = 51.72$, $p < 0.01$, $r^2 = 0.51$). ANCOVA for individuals greater than 15 years old showed a significant effect of age on killing capacity ($F_{1,35} = 4.49$, $p = 0.04$, $r^2 = 0.12$) (Figure 5). ANCOVA for individuals less than 15 years old also showed a significant effect of both site (Minnetaga versus all other sites combined) ($F_{1,51} = 59.47$, $p < 0.01$, $r^2 = 0.55$) and age ($F_{1,51} = 4.28$, $p = 0.04$, $r^2 = 0.04$). Note that an interaction term was not significant in the full ANCOVA, so we eliminated it in the post-hoc reduced ANCOVA with only additive effects. There was a significant negative correlation between telomere length in blood and bacteria-killing capacity (R = -0.27, n = 70, p = 0.03), but no correlation between killing capacity and telomere length from muscle (R = 0.09, n = 20, p = 0.51) or gonad (R = -0.15, n = 16, p = 0.58). NLR was not significantly correlated with bacteria-killing capacity (R = 0.15, n = 59, p = 0.25).

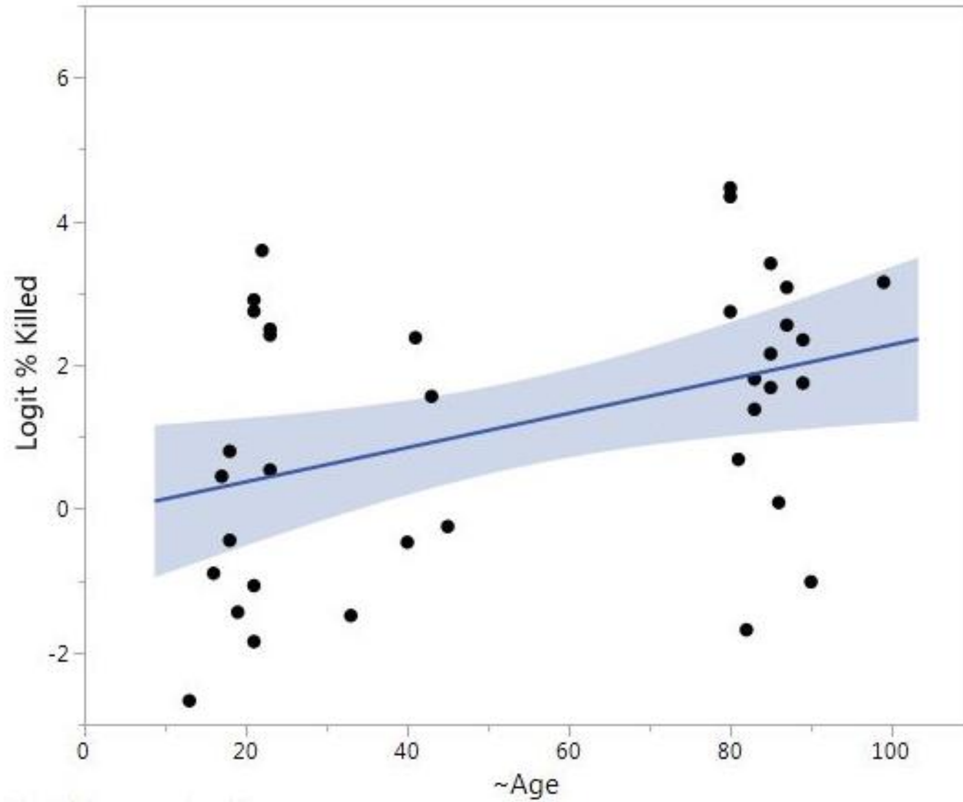

Figure 5. Age and logit-transformed bacteria killing capacity from bigmouth buffalo individuals 15 years of age or older.

Discussion

Neutrophil/Lymphocyte Ratio

In general, NLR has been shown to increase with chronic stress or disease (reviewed by Davis *et al.* 2008). The ratio of neutrophils to lymphocytes is significantly related to glucocorticoid levels, which in turn increase in vertebrates as stress exposure increases. In bigmouth buffalo, NLRs were significantly lower (i.e., the number of neutrophils decreased relative to the number of lymphocytes) in older fish (Figure 3). There is a well-documented increase in NLR in response to stress in vertebrates (Davis *et al.* 2008), and our findings suggest that older bigmouth buffalo generally experience lower levels of chronic stress than younger individuals. The stress of predator exposure in smaller, younger fish may explain the observed

difference in leukocyte profiles. Large, adult bigmouth buffalo have no natural predators (excluding humans) (Johnson 1963) and thus, may exhibit lower markers for chronic stress. NLR is also known to increase with age (Li *et al.* 2015, Satue *et al.* 2009). Lower stress levels in older individuals also indicates them to have higher fitness than younger fish. Chronic stress negatively impacts fitness. In birds, chronic stress evidenced by a low number of lymphocytes has been associated with increased susceptibility to disease (Al-Murrani *et al.* 2002), slow growth (Moreno *et al.* 2002), and even survival rates (Lobato *et al.* 2005). The oldest bigmouth buffalo had the highest lymphocyte counts (Figure 3), suggesting higher fitness than younger individuals. Our results contradict the patterns observed in terrestrial vertebrates in which higher NLR occur in older individuals.

An age-related decline in overall leukocyte production could explain the pattern we observed in bigmouth buffalo. If the production of neutrophils decreases at a greater rate than the production of lymphocytes, the NLR would decrease even though overall production of neutrophils and lymphocytes decreased as well. Indeed, the number of microscope viewing fields required to count 100 leukocytes (per the protocol for determining the NLR) increased with age. Using the number of microscope viewing fields to estimate cell density is limited, however our findings are consistent with the hypothesis that leukocyte production declines with age in bigmouth buffalo.

Chronic stress has been shown to reduce telomere length in vertebrates (Epel *et al.* 2004, Tyrka *et al.* 2010, Geiger *et al.* 2012, reviewed by Houben *et al.* 2008,). Our data also indicated that individuals with higher NLR had shorter red blood cell telomeres. Thus, individuals expressing markers positively related to exposure to chronic stress had shorter telomeres, which is consistent with the consensus of findings in other vertebrates. We did not see this signal in

muscle and gonad telomeres, but the sample sizes were smaller for these tissues. Although chronic stress explained a significant amount of variation in red blood cell telomere length in bigmouth buffalo, age did not, which is not consistent with observations in terrestrial vertebrates. Therefore, extrinsic factors such as environmental stress may play a larger role in telomere dynamics in bigmouth buffalo and other long-lived fishes, especially if these species have mechanisms (e.g., telomerase production) for preventing telomere deterioration caused by intrinsic factors.

Telomere Length

Telomere length shortens with age in terrestrial vertebrates, but telomerase activity may alter this pattern in fish. Fish exhibit continuous, but asymptotic growth. Shorter telomeres in larger (and presumably older) fish were not evident in common carp (*Cyprinus carpio*) (Izzo *et al.* 2014). Common carp and bigmouth buffalo share the same order (Cypriniformes) and habitats in North America, and have similar life histories (including lifespans) (Sanz *et al*. 2013). Our findings for bigmouth buffalo are consistent with those of Izzo *et al.* (2014) for common carp. According to the telomere hypothesis, cellular replication leads to telomere shortening, but telomerase, a reverse transcriptase, adds base pairs to restore telomeres. Anchelin *et al.* found that telomerase-deficient zebrafish exhibited shorter telomere lengths and premature aging symptoms compared to normal, wild-type zebrafish (2013). Telomerase may diminish senescence through telomere regulation, and high telomerase activity is observed in fishes (Anchelin *et al.* 2011, Hartmann *et al.* 2009, Hatakeyama *et al.* 2016, Klapper *et al.* 1998). Measuring telomerase levels within individuals is needed to determine if telomerase plays a significant role in determining telomere length in bigmouth buffalo and other long-lived fishes.

Telomere lengths were consistent for DNA extracted from different tissues in bigmouth buffalo. We found that red blood cell telomere lengths were significantly correlated with muscle telomere lengths, and approached ($p = 0.06$ for a sample size of 14) a significant correlation with gonad telomere lengths. Thus, if an individual had relatively long telomeres in DNA from red blood cells, we would expect the individual to have relatively long telomeres in DNA from muscle or gonad cells. That is, telomere lengths throughout different tissues within an individual bigmouth buffalo are relatively similar. The correlation between three tissues suggests that any trend observed in our large sample of red blood cell telomere lengths is likely to occur in other tissues. Our findings suggest that telomere measurements from red blood cells can offer general insight into telomere patterns in other tissues in bigmouth buffalo.

In addition, we found that telomere lengths from gonadal tissue and muscle tissue were significantly longer than telomere lengths from red blood cells. This result is consistent with prior works which found that telomere length in sperm was significantly longer than telomere length in normal somatic cells (Cook and Smith 1986) and that telomere length is maintained in human germline cells (Harley *et al.* 1990). Our results suggest that telomere regulation or starting telomere lengths can be tissue-dependent in bigmouth buffalo. Previous research has demonstrated that telomerase activity can differ across tissues (Prowse and Greider 1995, Klapper *et al.* 1998). Differences in telomerase activity among tissues could drive the observed differences in telomere length among gonad, muscle, and red blood cells in bigmouth buffalo. The initial telomere length could also vary across tissues. The rate of telomere loss could be similar throughout tissues, but gonadal tissue could simply begin with longer telomeres. Further research on initial lengths and rate of loss in telomeres from different tissues of bigmouth buffalo is needed to determine the underlying mechanism. Regardless of the mechanism, our observation

of longer telomeres in gonadal tissue indicates increased investment. For instance, reproductive cells may be protected from oxidative damage. Santiso *et al.* (2010) found that human sperm cells with longer telomeres were more successful at swim-up, and speculates there is selection for sperm with longer telomeres. Protection or restoration of telomeres in reproductive tissues could be one way in which bigmouth buffalo maintain reproductive fitness to the oldest ages of their lifespan.

The disposable soma theory suggests that organisms adjust their investment of resources between reproduction, self-maintenance, and growth (Kirkwood 1992). Accordingly, the optimum path is to invest the fewest resources into maintenance of somatic tissues that are only necessary for indeterminate survival, and to maximize resource allocation into reproduction. However, bigmouth buffalo appear to continue to invest in somatic maintenance long after sexual maturation. In terms of natural selection, this life-history strategy should act disadvantageously because it consumes resources that could be used for reproduction (Kirkwood 1992). However, in indeterminate growers, there may be advantages to continuing to invest in somatic tissue. Larger individuals produce more gametes and thereby increase potential reproductive output. If the benefits gained by reaching larger size exceed the costs of investing into somatic maintenance, there would be selection for continual investment into growth and longevity. This hypothesis is supported by evidence that the oldest individuals continue to produce viable gametes (Lackmann *et al*., in review). The bigmouth buffalo reproductive strategy may require repeated attempts at reproducing throughout life, especially when an individual is older and larger and reproductive potential is at its greatest, to take advantage of fortuitous seasons for offspring recruitment that may occur infrequently (Winemiller & Rose 1992).

Longitudinal studies have shown that individuals that start life with relatively long telomeres have higher fitness and survive to the oldest ages (Haussmann and Mauck 2008, Salomons *et al.* 2009). If this is the case for bigmouth buffalo, then our observations could have occurred if a large proportion of individuals had relatively long telomeres when spawned, and these individuals survived to old age because selection pressures favor longer telomeres (and telomere maintenance or restoration need not occur). However, if selection were acting on telomere length, we would expect the variation in telomere length to decrease at older ages, which we did not observe. Indeed, we observed significant variation in telomere lengths at all ages (Figure 4).

Our observations on telomere length variation in bigmouth buffalo do support Finch's (1994) hypothesis of negligible senescence. We observed many old individuals with long telomeres (Figure 4), and many old individuals with high condition values. In natural populations, the majority of adults do not survive to ages where senescence is expressed (Finch 1994). However, bigmouth buffalo grow indeterminately and likely are exposed to little predation risk after they reach 15-20 years (Figure 2). This suggests an intrinsic mechanism of mortality, but, unlike terrestrial vertebrates, telomere length does not appear to regulate the mechanism. Anchelin *et al.* (2011) reported telomere lengthening in the first 18 months of the three-year zebrafish lifespan, but a sharp decline in telomere length at 24 months. Certain teleost species may exhibit terminal senescence, in which senescence is delayed until the final stages of the lifespan, at which point rapid senescence occurs. This is pattern would not be detected if our sample of fish did not include any individuals nearing the end of their lifespan. Still, telomere length from relatively old individuals we collected suggests that senescence is negligible in individuals over 80 years old.

Bacteria Killing

Furthermore, the ability of plasma immune constituents to kill a common bacteria improved with age in bigmouth buffalo. Relatively old individuals not only failed to display the age-related decline in immune function observed in other vertebrates (including humans), but displayed increased killing capacity relative to younger individuals (Figure 5). In humans, the immune system exhibits progressive development. Newborns begin with an immature and weak immune system which develops and matures throughout infancy and childhood until reaching its optimum in adulthood. The immune system then begins to decline in old age, leading to major impacts on human health and mortality (Simon *et al.* 2015). While fish and humans share some similarities in physiology, the immune system of fish has different mechanistic pathways than mammalian immune system. The thymus regulates immune response of fishes and other non-mammalian vertebrates. However, contrary to more derived vertebrates, the size of the thymus varies with season as well as hormone levels in fish (Press & Evensen 1999). Furthermore, there is great diversity in the morphology of the immune system cells across the 20,000+ teleost species, adding further complexity to understanding the immune system in fishes (Press & Evensen 1999). Still, our findings for bigmouth buffalo indicate that senescence of the immune system is not observed in individuals of 90+ years old, and even suggest that the immune system of these individuals is operating at its optimum (Figure 5).

The energetic trade-off between reproductive investment, growth, and immune function may provide some insight into why we observed older fish with stronger immune function. Younger bigmouth buffalo grow at a much faster rate than older individuals (Figure 2), and may invest relatively more resources into somatic growth, which could limit their investment in immune constituents. Furthermore, older individuals may have accumulated exposure to a

greater number of pathogens and as a consequence acquired more antibodies or immunoglobulins, which is consistent with the variation we observed in killing capacity and age as well as sites because individuals from sites with more pathogens would also have greater accumulated exposure. Future research could look for correlations between killing capacity and pathogen abundance and diversity at site of collection.

Much like in telomeres, bigmouth buffalo life history strategy may explain investment in the immune system. Continued investment into immunity represents investment in potential future reproductive attempts, which is characteristic of species exploiting episodic, fortuitous reproductive seasons (Winemiller 1992, Winemiller & Rose 1992). Similar to somatic maintenance, immune function upkeep may be a mechanism that enables bigmouth buffalo to survive to old ages and sizes where reproductive potential is greatest. Although terminal senescence of immune function in bigmouth buffalo may eventually occur, our findings demonstrate that fish nearing 100 years old are not experiencing immunosenescence.

Despite the association of shorter telomere length with weak immune function (Roberts-Thomson *et al.* 1974, Katepalli *et al.* 2008), we found a negative correlation between telomere length and immune function in bigmouth buffalo. Individuals with longer telomeres from red blood cells had plasma less effective at inhibiting bacterial growth than individuals with shorter telomeres. This result contradicts the current understanding of telomere length and immune function. However, we did not analyze telomere length in cells that are associated with the immune system. While telomere length could be regulated separately in immune cells, telomere lengths of DNA from red blood cells was consistent with lengths from DNA in muscle and gonad. Normal lymphocytes generally express telomerase suggesting that telomere regulation may be an important part of maintaining function of T and B cells (Hodes *et al.* 2002). Future

research exploring the variation between immune function and telomere length in immune cells or between telomere length of erythrocytes and leukocytes of long-lived fish such as bigmouth buffalo would be helpful in elucidating cellular mechanisms maintaining immune constituents.

Conclusions

Telomere dynamics in bigmouth buffalo, a long-lived teleost fish, exhibit unique and often contradictory patterns compared to the dynamics seen in terrestrial vertebrates. Telomere length was not related to age, and yet lengths were consistent in three different tissues (humoral, muscular and gonadal). Chronic stress explained some of the variation in telomere length, but telomere dynamics in bigmouth buffalo remain perplexing. Prior research on telomere dynamics in different fish species has not provided a general framework. However, prior to our study, almost no information was available on age and telomere dynamics in fish over 10 years old. Our findings were consistent with findings from Izzo *et al.* (2014) on telomere dynamics with size. Izzo *et al.* (2014) did not determine age in the common carp used in their study, but based on size the largest individuals were likely over 20 years of age, and common carp have a comparable, but shorter, lifespan than bigmouth buffalo (Lackmann *et al*. in review). Thus, we hypothesize that telomeres do not shorten with age in long-lived fish, and that these species have an intrinsic mechanism of telomere maintenance.

We found no evidence of age-related decline in bigmouth buffalo that were approaching 100 years old. NLR decreased with age and immune function improved with age, both observations that contradict the typical path of age-related senescence observed in humans and other vertebrates. Finch and Austad (2001) described the criteria for negligible senescence to include a lack of age-related increase in mortality or decrease in reproduction, as well as a lack of age-related decline in physiological capacity or disease resistance. Our data are consistent

with these criteria and provide evidence for negligible senescence in the bigmouth buffalo. Negative senescence is characterized by increased fecundity, decreased mortality, and improved function with age (Vaupel *et al*. 2004). Some of the patterns we observed could emerge with negative senescence. However, our findings are based on cross-sectional rather than longitudinal observations of individuals, which masks individual telomere length dynamics (Dunshea *et al.* 2011) because there is variation among individuals in initial telomere lengths, rates of loss and restoration, telomerase levels, and rates of cellular turnover (Monaghan and Haussmann 2006). Longitudinal studies within individuals are needed at a species-specific level to help clarify telomere dynamics and senescence in long-lived fish. Nevertheless, our findings for bigmouth buffalo provided consistent evidence of negligible senescence using different metrics in the oldest freshwater teleost fish (Lackmann *et al.* in review). Telomere dynamics in species that do not exhibit senescence can help us understand the mechanistic explanations for apparent immortality.

REFERENCES

Adámek, Z., Sukop, I., Rendón, P. M., & Kouřil, J. (2003). Food competition between 2+ tench (Tinca tinca L.), common carp (Cyprinus carpio L.) and bigmouth buffalo (Ictiobus cyprinellus Val.) in pond polyculture. *Journal of Applied Ichthyology*, *19*(3), 165-169.

Al-Murrani, W. K., Al-Rawi, I. K., & Raof, N. M. (2002). Genetic resistance to Salmonella typhimurium in two lines of chickens selected as resistant and sensitive on the basis of heterophil/lymphocyte ratio. *British poultry science*, *43*(4), 501-507.

Anchelin, M., Murcia, L., Alcaraz-Pérez, F., García-Navarro, E. M., & Cayuela, M. L. (2011). Behaviour of telomere and telomerase during aging and regeneration in zebrafish. *PloS one*, *6*(2), e16955.

Anchelin, M., Alcaraz-Pérez, F., Martínez, C. M., Bernabé-García, M., Mulero, V., & Cayuela, M. L. (2013). Premature aging in telomerase-deficient zebrafish. *Disease models & mechanisms*, dmm-011635.

Andrews, N. P., Fujii, H., Goronzy, J. J., & Weyand, C. M. (2010). Telomeres and immunological diseases of aging. *Gerontology*, *56*(4), 390-403.

Aubert, G., & Lansdorp, P. M. (2008). Telomeres and aging. *Physiological reviews*, *88*(2), 557-579.

Bennett, M. F., & Neville, C. G. (1975). Effects of cold shock on the distribution of leucocytes in goldfish, Carassius auratus. *Journal of comparative physiology*, *98*(2), 213-216.

Bly, J. E., Miller, N. W., & Clem, L. W. (1990). A monoclonal antibody specific for neutrophils in normal and stressed channel catfish. *Developmental & Comparative Immunology*, *14*(2), 211-221.

Bronikowski, A. M. (2008). The evolution of aging phenotypes in snakes: a review and synthesis with new data. *Age*, *30*(2-3), 169.

Cooke, H. J., & Smith, B. A. (1986, January). Variability at the telomeres of the human X/Y pseudoautosomal region. In *Cold Spring Harbor symposia on quantitative biology* (Vol. 51, pp. 213-219). Cold Spring Harbor Laboratory Press.

Damjanovic, A. K., Yang, Y., Glaser, R., Kiecolt-Glaser, J. K., Nguyen, H., Laskowski, B., & Weng, N. P. (2007). Accelerated telomere erosion is associated with a declining immune function of caregivers of Alzheimer's disease patients. *The Journal of Immunology*, *179*(6), 4249-4254.

Davis, A. K., Maney, D. L., & Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology*, *22*(5), 760-772.

Dethloff, G. M., Schlenk, D., Hamm, J. T., & Bailey, H. C. (1999). Alterations in physiological parameters of rainbow trout (Oncorhynchus mykiss) with exposure to copper and copper/zinc mixtures. *Ecotoxicology and Environmental Safety*, *42*(3), 253-264.

Dick, P. T., & Dixon, D. G. (1985). Changes in circulating blood cell levels of rainbow trout, Salmo gairdneri Richardson, following acute and chronic exposure to copper. *Journal of Fish Biology*, *26*(4), 475-481.

Dunshea, G., Duffield, D., Gales, N., Hindell, M., Wells, R. S., & Jarman, S. N. (2011). Telomeres as age markers in vertebrate molecular ecology. *Molecular Ecology Resources*, *11*(2), 225-235.

Ellsaesser, C. F., & Clem, L. W. (1986). Haematological and immunological changes in channel catfish stressed by handling and transport. *Journal of Fish Biology*, *28*(4), 511-521.

Epel, E. S., Blackburn, E. H., Lin, J., Dhabhar, F. S., Adler, N. E., Morrow, J. D., & Cawthon, R. M. (2004). Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences*, *101*(49), 17312-17315.

di Fagagna, F. D. A., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., von Zglinicki, T., ... & Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature*, *426*(6963), 194.

Finch, C. E. (1994). *Longevity, senescence, and the genome*. University of Chicago Press.

Finch, C. E. (1998). Variations in senescence and longevity include the possibility of negligible senescence. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *53*(4), B235-B239.

Finch, C. E., & Austad, S. N. (2001). History and prospects: symposium on organisms with slow aging. *Experimental gerontology*, *36*(4-6), 593-597.

French, S. S., DeNardo, D. F., Greives, T. J., Strand, C. R., & Demas, G. E. (2010). Human disturbance alters endocrine and immune responses in the Galapagos marine iguana (Amblyrhynchus cristatus). *Hormones and behavior*, *58*(5), 792-799.

Geiger, S., Le Vaillant, M., Lebard, T., Reichert, S., Stier, A., Le Maho, Y., & Criscuolo, F. (2012). Catching-up but telomere loss: half-opening the black box of growth and ageing trade-off in wild king penguin chicks. *Molecular Ecology*, *21*(6), 1500-1510.

Gopalakrishnan, S., Cheung, N. K., Yip, B. W., & Au, D. W. (2013). Medaka fish exhibits longevity gender gap, a natural drop in estrogen and telomere shortening during aging: a unique model for studying sex-dependent longevity. *Frontiers in zoology*, *10*(1), 78.

Greeley, E. H., Ballam, J. M., Harrison, J. M., Kealy, R. D., Lawler, D. F., & Segre, M. (2001). The influence of age and gender on the immune system: a longitudinal study in Labrador Retriever dogs. *Veterinary immunology and immunopathology*, *82*(1-2), 57-71.

Greider, C. W. (1998). Telomerase activity, cell proliferation, and cancer. *Proceedings of the National Academy of Sciences*, *95*(1), 90-92.

Harley, C. B., Futcher, A. B., & Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature*, *345*(6274), 458.

Hartmann, N., Reichwald, K., Lechel, A., Graf, M., Kirschner, J., Dorn, A., ... & Cellerino, A. (2009). Telomeres shorten while Tert expression increases during ageing of the short-lived fish Nothobranchius furzeri. *Mechanisms of ageing and development*, *130*(5), 290-296.

Hatakeyama, H., Yamazaki, H., Nakamura, K. I., Izumiyama-Shimomura, N., Aida, J., Suzuki, H., & Ishikawa, N. (2016). Telomere attrition and restoration in the normal teleost Oryzias latipes are linked to growth rate and telomerase activity at each life stage. *Aging (Albany NY)*, *8*(1), 62.

Hatase, H., Sudo, R., Watanabe, K. K., Kasugai, T., Saito, T., Okamoto, H., & Tsukamoto, K. (2008). Shorter telomere length with age in the loggerhead turtle: a new hope for live sea turtle age estimation. *Genes & genetic systems*, *83*(5), 423-426.

Haussmann, M. F., & Mauck, R. A. (2008). TECHNICAL ADVANCES: New strategies for telomere-based age estimation. *Molecular Ecology Resources*, *8*(2), 264-274.

Haussmann, M. F., & Vleck, C. M. (2002). Telomere length provides a new technique for aging animals. *Oecologia*, *130*(3), 325-328.

Haussmann, M. F., Winkler, D. W., O'Reilly, K. M., Huntington, C. E., Nisbet, I. C., & Vleck, C. M. (2003). Telomeres shorten more slowly in long-lived birds and mammals than in short–lived ones. *Proceedings of the Royal Society of London B: Biological Sciences*, *270*(1522), 1387-1392.

Heidinger, B. J., Herborn, K. A., Granroth-Wilding, H. M., Boner, W., Burthe, S., Newell, M., ... & Monaghan, P. (2016). Parental age influences offspring telomere loss. *Functional Ecology*, *30*(9), 1531-1538.

Hodes, R. J., Hathcock, K. S., & Weng, N. P. (2002). Telomeres in T and B cells. *Nature Reviews Immunology*, *2*(9), 699.

Horn, T., Robertson, B. C., & Gemmell, N. J. (2010). The use of telomere length in ecology and evolutionary biology. *Heredity*, *105*(6), 497.

Houben, J. M., Moonen, H. J., van Schooten, F. J., & Hageman, G. J. (2008). Telomere length assessment: biomarker of chronic oxidative stress?. *Free radical biology and medicine*, *44*(3), 235-246.

Iwama, G. K. (1998). Stress in fish. *Annals of the New York Academy of Sciences*, *851*(1), 304-310.

Izzo, C., Bertozzi, T., Gillanders, B. M., & Donnellan, S. C. (2014). Variation in telomere length of the common carp, Cyprinus carpio (Cyprinidae), in relation to body length. *Copeia*, *2014*(1), 87-94.

Johnson, R. P. (1963). Studies on the life history and ecology of the bigmouth buffalo, Ictiobus cyprinellus (Valenciennes). *Journal of the Fisheries Board of Canada*, *20*(6), 1397-1429.

Kaszubowska, L. (2008). Telomere shortening and ageing of the immune system. *J Physiol Pharmacol*, *59*(Suppl 9), 169-186.

Katepalli, M. P., Adams, A. A., Lear, T. L., & Horohov, D. W. (2008). The effect of age and telomere length on immune function in the horse. *Developmental & Comparative Immunology*, *32*(12), 1409-1415.

Kirkwood, T. B. L. (1992). The disposable soma theory: evidence and implications. *Netherlands journal of zoology*, *43*(3), 359-363.

Klapper, W., Heidorn, K., Kühne, K., Parwaresch, R., & Krupp, G. (1998). Telomerase activity in 'immortal'fish 1. *FEBS letters*, *434*(3), 409-412.

Kotrschal, A., Ilmonen, P., & Penn, D. J. (2007). Stress impacts telomere dynamics. *Biology letters*, *3*(2), 128-130.

Li, J., Chen, Q., Luo, X., Hong, J., Pan, K., Lin, X., & Li, H. (2015). Neutrophil-to-lymphocyte ratio positively correlates to age in healthy population. *Journal of clinical laboratory analysis*, *29*(6), 437-443.

Lobato, E., Moreno, J., Merino, S., Sanz, J. J., & Arriero, E. (2005). Haematological variables are good predictors of recruitment in nestling pied flycatchers (Ficedula hypoleuca). *Ecoscience*, *12*(1), 27-34.

Makinodan, T., & Kay, M. M. (1980). Age influence on the immune system. In *Advances in immunology* (Vol. 29, pp. 287-330). Academic Press.

Monaghan, P., & Haussmann, M. F. (2006). Do telomere dynamics link lifestyle and lifespan?. *Trends in Ecology & Evolution*, *21*(1), 47-53.

Moreno, J., Merino, S., MartÍnez, J., Sanz, J., & Arriero, E. (2002). Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Ecoscience*, *9*(4), 434-439.

Press, C. M., & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish & Shellfish Immunology*, *9*(4), 309-318.

Prowse, K. R., & Greider, C. W. (1995). Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proceedings of the National Academy of Sciences*, *92*(11), 4818-4822.

Roberts-Thomson, I., Youngchaiyud, U., Whittingham, S., & Mackay, I. (1974). Ageing, immune response, and mortality. *The Lancet*, *304*(7877), 368-370.

Rollings, N., Miller, E., & Olsson, M. (2014). Telomeric attrition with age and temperature in Eastern mosquitofish (Gambusia holbrooki). *Naturwissenschaften*, *101*(3), 241-244.

Salomons, H. M., Mulder, G. V., van de Zande, L., Haussmann, M. F., Linskens, M. H., & Verhulst, S. (2009). Telomere shortening and survival in free-living corvids. *Proceedings of the Royal Society of London B: Biological Sciences*, *276*(1670), 3157-3165.

Santiso, R., Tamayo, M., Gosálvez, J., Meseguer, M., Garrido, N., & Fernández, J. L. (2010). Swim-up procedure selects spermatozoa with longer telomere length. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *688*(1), 88-90.

Sanz, A., Trenzado, C. E., Castro, H. B., López-Rodríguez, M. J., & de Figueroa, J. T. (2013). Relationship between brain and liver oxidative state and maximum lifespan potential of different fish species. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *165*(3), 358-364.

Satue, K., Blanco, O., & Munoz, A. (2009). Age-related differences in the hematological profile of Andalusian broodmares of Carthusian strain. *Veterinarni Medicina*, *54*(4), 175-182.

Scott, N. M., Haussmann, M. F., Elsey, R. M., Trosclair III, P. L., & Vleck, C. M. (2006). Telomere length shortens with body length in Alligator mississippiensis. *Southeastern Naturalist*, *5*(4), 685-692.

Simon, A. K., Hollander, G. A., & McMichael, A. (2015). Evolution of the immune system in humans from infancy to old age. *Proc. R. Soc. B*, *282*(1821), 20143085.

Tsuji, A., Ishiko, A., Takasaki, T., & Ikeda, N. (2002). Estimating age of humans based on telomere shortening. *Forensic Science International*, *126*(3), 197-199.

Tyrka, A. R., Price, L. H., Kao, H. T., Porton, B., Marsella, S. A., & Carpenter, L. L. (2010). Childhood maltreatment and telomere shortening: preliminary support for an effect of early stress on cellular aging. *Biological psychiatry*, *67*(6), 531-534.

Utsuyama, M., Kasai, M., Kurashima, C., & Hirokawa, K. (1991). Age influence on the thymic capacity to promote differentiation of T cells: induction of different composition of T cell subsets by aging thymus. *Mechanisms of ageing and development*, *58*(2-3), 267-277.

Valenzuela, A. E., Silva, V. M., & Klempau, A. E. (2008). Effects of different artificial photoperiods and temperatures on haematological parameters of rainbow trout (Oncorhynchus mykiss). *Fish physiology and biochemistry*, *34*(2), 159-167.

Vaupel, J. W., Baudisch, A., Dölling, M., Roach, D. A., & Gampe, J. (2004). The case for negative senescence. *Theoretical population biology*, *65*(4), 339-351.

Vleck CM, Vertalino N, Vleck D, Bucher TL (2000) Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adelie Penguins. *Condor* 102: 392-400

von Zglinicki, T. (2002). Oxidative stress shortens telomeres. *Trends in biochemical sciences*, *27*(7), 339-344.

Winemiller, K. O. (1992). Life-history strategies and the effectiveness of sexual selection. *Oikos* 63: 318-327

Winemiller, K. O., Rose, K.A. (1992). Patterns of life-history diversification in North American fishes: implications for population regulation. *Canadian Journal of Fisheries and Aquatic Sciences* 49: 2196-2218

Witeska, M. (2005). Stress in fish-hematological and immunological effects of heavy metals. *Electronic Journal of Ichthyology*, *1*(1), 35-41.

Zysling, D. A., Garst, A. D., & Demas, G. E. (2009). Photoperiod and food restriction differentially affect reproductive and immune responses in Siberian hamsters Phodopus sungorus. *Functional Ecology*, *23*(5), 979-988.