Patterns of telomere length change with age in aquatic vertebrates and the phylogenetic distribution of the pattern among jawed vertebrates

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“A gentle way to age”

…Carina Dennis (2006)

Declaration

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Izzo, C., Bertozzi, T., Donnellan, C. E. & Gillanders, B. M. (*In prep*). Telomere length varies independently of age in the Port Jackson shark, *Heterodontus portusjacksoni*: with a commentary on telomere length methods.

Izzo, C., Bertozzi, T, Gillanders, B. M. & Donnellan, C. E. (*In prep*). Variation in telomere length of the common carp, *Cyprinus carpio* (Cyprinidae) in relation to age and tissue type.

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Izzo, C., Gillanders, B. M. & Donnellan, C. E. (*In prep*). Telomere length of fishes correlates with variation in longevity.

Izzo, C., Donnellan, C. E. & Gillanders, B. M. (*In prep*). Recent evolution of telomere length change with age in gnathostomes.

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Christopher Izzo

April 2010

“Bad times have a scientific value.

These are occasions a good learner

would not miss”

…Ralph Waldo Emerson (1837)

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Thesis Abstract

Telomeres, the protective caps at the ends of all vertebrate chromosomes, naturally undergo changes in length. These changes in telomere lengths may be a "molecular clock" by providing a counting mechanism of DNA replication events. In populations of jawed vertebrates (gnathostomes), telomere length has been shown to change with age; and thus measurements of telomere lengths may provide a novel means of determining the ages of free-living animals. Determinations of the age structure of populations of aquatic vertebrates (teleosts, chondrichthyans and marine mammals) are vital for sustainable management and conservation efforts. Yet, the commonly applied increment based ageing techniques are limited by the subjectivity of increment patterning and destructive sampling. I aimed to assess the application of telomere length as an age determinate for populations of aquatic vertebrates and to evaluate the biological implications and evolutionary origins of this trait amongst the gnathostomes.

Telomere length change with age was investigated in an exemplar chondrichthyan, teleost and marine mammal species, to determine whether aquatic gnathostomes share the general pattern of declining telomeres with age, as found in terrestrial mammals. Chapter Two provides the first assessment of telomere length change with age in a chondrichthyan species, the Port Jackson shark. Four types of tissues from Port Jackson sharks, ranging in age from 0 to 17 years, were sampled and telomere length were estimated using three measurement methods: (i) relative quantitative PCR (qPCR); (ii) absolute qPCR; and (iii) the terminal restriction fragment (TRF) analysis. No relationship between telomere length and age was found for any of the tissues, using any method.

In Chapter Three, telomere length was measured in specimens of the common carp from two tissues using the absolute qPCR method. Telomere length measurements were then correlated with ages estimated from otolith increment counts and length-at-age calculations. Measurements of telomere length were highly variable in both muscle biopsies and fin clips; however, telomeres from muscle biopsies significantly increased in length – in contrast to the more generalised pattern of telomere length attrition and marking the second reported case of an increase in telomere length with age in vertebrates.

In terrestrial mammals telomere shortening is negatively correlated with donor age. In Chapter Four, I tested whether this pattern of declining telomere lengths was found in a pinniped species, the Australian sea lion. Telomere lengths were measured in flipper clips from specimens by absolute qPCR and compared between three age classes: pups, juveniles, and adults. Mean telomere lengths of the adults were significantly smaller than the juvenile and pup classes confirming that the Australian sea lion shares the general mammalian pattern of telomere length attrition.

Relationships between the rate of telomere length change with age and species longevity have been observed in birds and mammals, suggesting that the rate of telomere length change is an informative measure of ageing. In Chapter Five, using a data set of 20 teleost and chondrichthyan species, I tested whether fishes showed a similar pattern. I found that the rate of telomere length change with age is significantly different between species of fishes and that these rates of change are inversely correlated with longevity.

The findings of Chapters Two, Three & Four indicated that telomeres do not provide a suitable means of determining the ages of individuals and at best are limited to assigning broad age classes. This is largely due to the high degree of variability of telomere lengths between individuals within all age classes. In addition, these Chapters (2, 3 & 4) also highlight that patterns of telomere length change with age are highly variable within the gnathostomes; and thus, telomere length change cannot be characterised by a single pattern for all lineages. In fact, three patterns of telomere length change with age in the gnathostomes were found: (i) declining telomere lengths; (ii) increasing telomere lengths; and (iii) no significant change in telomere length with age. However, identifying the selective factors responsible for the assignment of patterns of telomere length change is hampered by a lack of the understanding of the evolutionary origins of these patterns.

Therefore, in Chapter Six I sought to outline the phylogenetic distribution of patterns of telomere length change with age in the gnathostomes to determine the evolutionary origin(s) of this trait. Two alternative hypotheses for the evolution of telomere length change were tested by ancestral state reconstruction in a set of 40 gnathostomes, for which I have significantly expanded the sampling of chondrichthyans and teleosts. The

most likely/parsimonious pattern of telomere length change in the common gnathostome ancestor suggested that telomere length change with age was not present ancestrally and has since evolved independently. I was also able to elucidate the evolutionary history of transitions to and between the three patterns of telomere length change within the available gnathostome lineages, with the birds and teleosts displaying the highest rates of evolutionary lability of patterns of telomere length change with age.

The macro-evolutionary analysis (Chapter 6) identified relatively rapid evolutionary patterns of telomere length change with age in two gnathostome clades. However, as highlighted by the variability of telomere lengths among individuals within all age classes, furthering an interpretation of the causes and consequences of variable patterns of telomere length change will require a focus at the species level and a shift to following individuals through out their lifetime.

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Thesis Preface

Note on Chapter Style

This thesis begins with a brief General Introduction (Chapter One) to telomeres and the factors that affect telomere length dynamics. The introduction also highlights the variable nature of telomere length change with age in the jawed vertebrates (gnathostomes) and the paucity of telomeric research in the teleostean and chondrichthyan lineages. Here the broad research objectives of the thesis will be established. The General Introduction is followed by five research chapters (Chapters Two to Six) addressing the outlined research objectives. Finally, the biological and evolutionary implications of the findings presented herein are addressed in the General Discussion (Chapter Seven).

The research chapters of this thesis have been written in a style suitable for publication in a scientific journal and can be read as separate studies, therefore there is some repetition in each introduction and in the methodological descriptions. Furthermore, as these chapters have co-authors they have been written in plural.

Each research chapter is preceded by a preface, which includes the chapter abstracts, to summarise the contents and guide the reader, as well as a brief preamble that presents information on the publication status of the chapter at the time of thesis submission, and describes the contributions of all co-authors to the research therein. Finally, the chapter acknowledgements are also provided.

All tables and figures appear embedded within the text and the numbering of figures and tables begins at one for each chapter to simplify referral to the results. All literature cited in the thesis chapter‟s have been compiled at the end of the thesis and not at the end of each chapter. Additional appendices provided are referred to in the text as appropriate.

“Picture an aglet…”

…the author

ag·let (ag ’lit) *n*. A tag or sheath, as of plastic, on the end of a lace, cord, or ribbon to facilitate its passing through eyelet holes and maintain end integrity

Chapter One

GENERAL INTRODUCTION: Patterns of telomere length change with age in aquatic vertebrates and the phylogenetic distribution of the pattern among jawed vertebrates

Accurate determinations of age are essential for providing information on fundamental life history parameters of aquatic vertebrates (teleosts, chondrichthyans, marine mammals, reptiles and birds), including population age structure, growth rates, longevity, age and size at maturity, fecundity and annual reproductive cycles (Pauly 1987; Hoenig & Gruber 1990; Quinn & Dersio 1999; Campana 2001). Age and growth data also form the basis for ecological risk assessments of exploited populations, which are increasingly used to identify species that require immediate management and, or conservation action (Duvley et al. 2004; Walker 2004; Braccini et al. 2006). Thus, methodologies for the accurate determination of age are an essential requirement for prudent management of exploited and endangered populations of aquatic vertebrates.

Increment based age determinations of aquatic vertebrates

Generally aquatic vertebrates are aged based on the enumeration of growth increments in calcified tissues (Campana 2001; Cailliet & Goldman 2004). These calcified tissues, the otoliths and scales of teleosts, the vertebral centra of chondrichthyans and the teeth of marine mammals, form growth increments as a result of the incorporation of dense calcium deposits during periods of rapid growth (Casselman 1983; Stewart et al. 1996). While this ageing technique has been applied widely to fish over the last 120 years (Quinn & Dersio 1999), it is time consuming, subject to error in terms of precision and accuracy, is not possible in some chondrichthyans, and generally requires the destructive sampling of specimens (Casselman 1983; Beamish & McFarlane 1987; Cailliet & Goldman 2004). The destructive sampling of specimens is particularly problematic for endangered or slow growing species with small population sizes (Camhi et al. 1998; Musick 1999). The limitations of this commonly used ageing method are

significant impediments towards the sustainable management of endangered and exploited populations of aquatic vertebrates. Therefore, there is clearly an urgent need for the development of a rapid, accurate and non-destructive methodology for ageing. Measurements of telomere lengths have been suggested as a novel and potentially non-lethal age determinate for free-living animals (Haussmann & Vleck 2002).

Telomeres

Telomeres are the physical ends of linear eukaryotic chromosomes (Fig. 1). In vertebrates, the telomeric repeats unit consists of a highly conserved, guanine (G) rich sequence of non-coding DNA $(TTAGGG)_n$ (Moyzis et al. 1988; Meyne et al. 1989; Zakian 1995). At the very ends of vertebrate telomeres, a single strand of G-rich DNA overhangs the telomeric ends (Huffman et al. 2000). In vertebrates, the overhang folds back in on itself and is re-incorporated into the double-stranded section, forming the "T–loop" (Griffith et al. 1999; Cech 2004). This, together with several specific and non-specific proteins, creates the telomeric cap at the end of chromosomes that is essential to telomere function (Biessmann & Mason 1994; Saldanha et al. 2003). This capping function of telomeres enables the cell to differentiate between real chromosome ends and those arising from chromosome breaks that need to be repaired (Blackburn 1990, 1991).

Figure 1. Telomeres (paired black dots) visualized by *in situ* hybridization of a telomere probe on human chromosome ends (image sourced from http://commons.wikimedia.org/wiki/File:Telomere_caps.gif).

Telomeres, along with associated proteins, play an essential part in DNA replication (Levy et al. 1992; Saldanha et al. 2003). Telomeres protect the encoding parts of the chromosomes from the loss of nucleotides that occurs because chromosome ends are not replicated completely during cell division (Biessmann & Mason 1994). Telomeres also stabilise chromosomes and facilitate the formation of stable structures that prevent DNA degradation and chromosome fusion, whilst anchoring chromosomes in the nuclear matrix (Blackburn 1991; Prowse & Greider 1995). Finally, telomeres appear to have a role in the alignment and segregation of chromosomes during meiosis (Biessmann & Mason 1994).

Telomere length change

Telomere length is kept at equilibrium by processes that shorten telomeres (the end-replication problem) and processes that lengthen telomeres (the enzymatic catalysis of telomere synthesis by telomerase) (Allsopp et al. 1995; Lingner & Cech 1998). The end-replication problem is associated with the loss of telomeric sequences upon the completion of cellular division (Blackburn 2000; Nosek et al. 2004). At each cell division, the unidirectional nature of DNA synthesis by DNA polymerase results in the incomplete replication of a small number of telomeric repeats at the extreme 3'–end of the linear chromosomes (Fig. 2). This results in the telomeric G–rich overhanging strand (Saldanha et al. 2003; Nosek et al. 2004). Telomere length change occurs during progressive cell divisions, which in turn are inherited by the daughter cells and the process repeats itself in subsequent divisions (Blasco et al. 1999; Nosek et al. 2004). Telomere loss puts a finite limit on the reproductive life of cells (Allsopp et al. 1992). Each time a cell divides, the telomeric DNA on its chromosomes gets shorter, unless it is restored (Blackburn 1990; von Zglinicki 2003).

Telomeres also become dysfunctional as a result of direct DNA damage and, or changes in their associated proteins (von Zglinicki et al. 2000). Telomeric impairment results in genomic instability, as chromosome ends become “uncapped”. The uncapping of chromosome ends results in the activation of the DNA repair mechanisms that recognise the region as a double-stranded break (Blackburn 2001). Generally, the repair of the double-stranded break results in one of two outcomes: cell death (apoptosis), or replicative senescence, whereby the cell continues to function but is incapable of dividing (Blackburn 2001; Hemann et al. 2001).

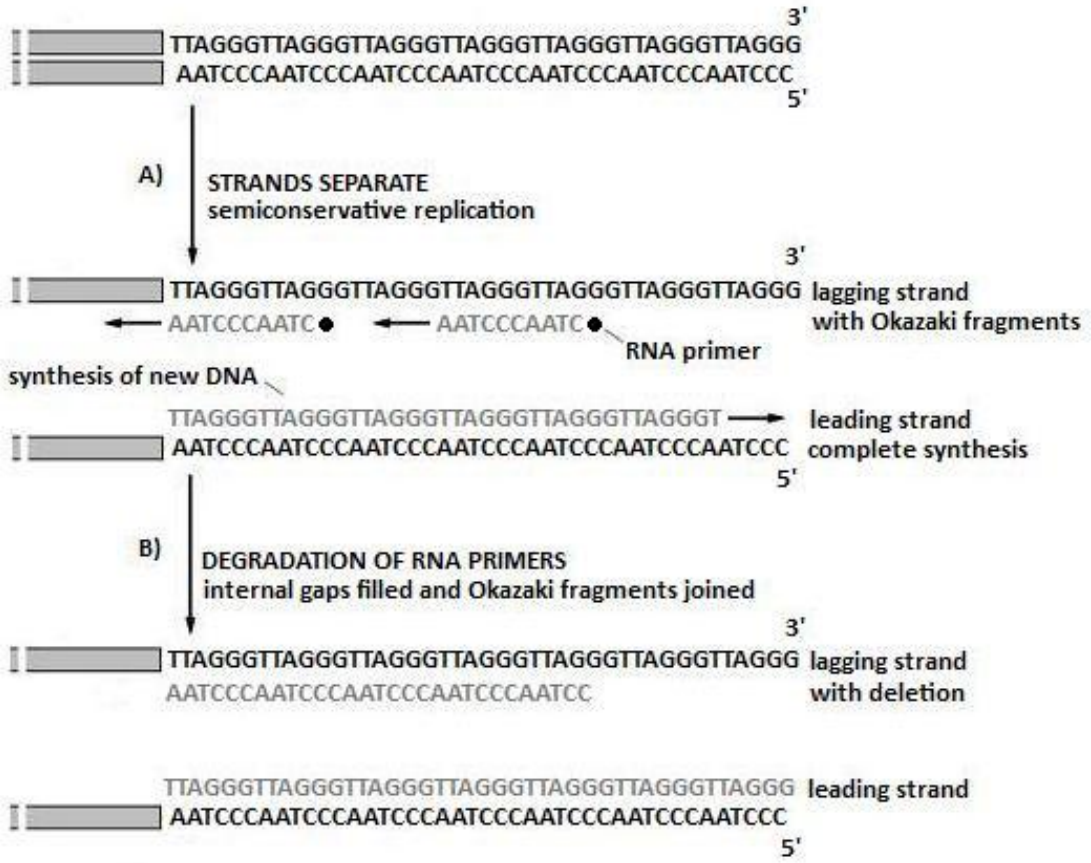

Figure 2. The end-replication problem at one end of linear DNA. A) Strand separation and the unidirectional (5'➞ 3') nature of the synthesis of new DNA leads to the staggered replication of the lagging strand. B) Okazaki fragments are initiated with an RNA primer (●), which is later degraded. Since only the internal gap can be filled, the 3'–end of the parental strand is left incompletely copied (adapted from Harley et al. 1992).

In order to compensate for telomere sequence loss, telomerase, a ribonucleoprotein enzyme, stabilizes telomere length by adding telomeric repeats onto the ends of the chromosomes (Fig. 3) (Venkatesan & Price 1998; Blasco et al. 1999). Telomerase is generally active in the germ line and in embryonic tissues of vertebrates, enabling the replicative potential of germ cells to persist, and can reset telomere length during early embryogenesis (Murnane et al. 1994; Lee at al. 1998). Yet, in *in vivo* and *in vitro* mammalian research, progressive telomere shortening is observed in somatic cells, which eventually leads to greatly shortened telomeres, limiting the chromosome˝s ability to continue dividing (Nosek et al. 2004). Once some crucial length is reached, the telomere becomes dysfunctional as the functional protective capping of chromosome ends is lost (Allsopp & Harley 1995; Hemann et al. 2001). When telomeres reach this critical length, cells enter a state of cellular senescence resulting in the cessation of normal cellular division, marking either apoptosis, or carcinogenesis (Campisi et al. 2001; Nosek et al. 2004).

These naturally occurring changes in telomere lengths have been proposed to act as a molecular "clock" that counts the number of times a cell has undergone cellular replication (Olovnikov 1973), thereby acting as a biomarker of biological aging and replicative senescence (Harley et al. 1992). As early as 1990, relationships between telomere length and chronological age were observed in *in vivo* and *in vitro* mammalian research (Hastie et al. 1990).

Taxonomic bias

I conducted a literature search using the Web of Science, an online academic database from ISI Web of Knowledge [http://apps.isiknowledge.com], which provides access to information from over 8,700 research journals. Articles published between 1987 and 2008 were searched (as of 4th December, 2008) for three terms: *telomer**, *ag** and *length** (where * denotes the inclusion of derivatives of the assigned term). The following document types were excluded: review articles (n = 102), meeting abstracts (n = 74), editorial material or letters (n = 15) and corrections or additions (n = 2).

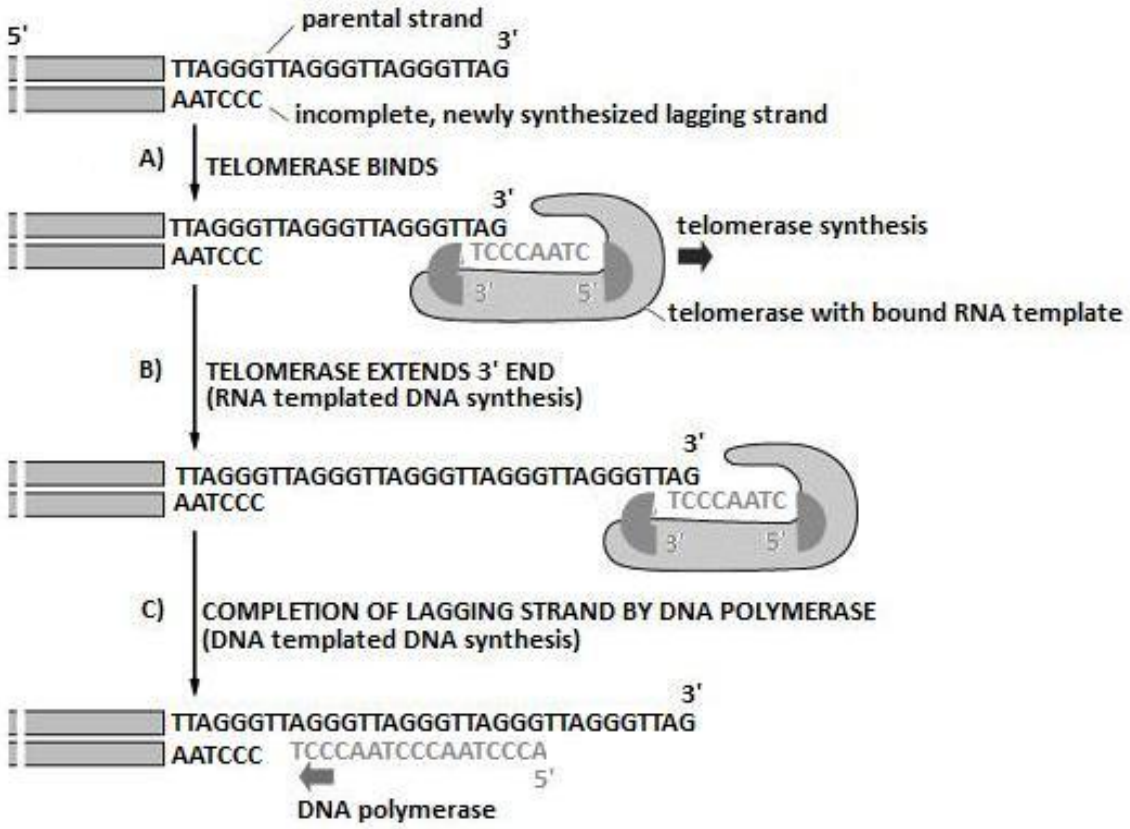

Figure 3. Telomerase and telomere length elongation. A) To extend the length of a telomere, the telomerase first extends its longer strand. Then, using the same mechanism as synthesizing the lagging strand, the shorter strand is extended. B) Using an internal RNA template, telomerase adds a repeating nucleotide sequence to the 3' strand of chromosomes. C) Telomerase binds the first few nucleotides of the RNA template to the last telomere sequence on the chromosome, add a new repeat sequence, detach, realign to the new 3'–end of telomere to the template, and repeat the process (adapted from Blackburn 1992; Autexier & Lue 2006).

The Web of Science literature search identified 777 research papers published between 1987 and 2008 that contained the terms *telomere*, *age* and *length* or derivatives of the three terms. Of the 777 research papers identified, the majority (79.7 %) investigated human telomeric biology *in vivo* and, or *in vitro* (Fig. 4). In total, 3 papers concerning fish telomeric biology were published over this period, which constituted 0.3 % of the total articles (Fig. 4). When using the same search engine, with the terms: *telomer** and *teleost*, and searching: *telomer** and *chondrichthy**, 13 and 3 published papers were identified, respectively.

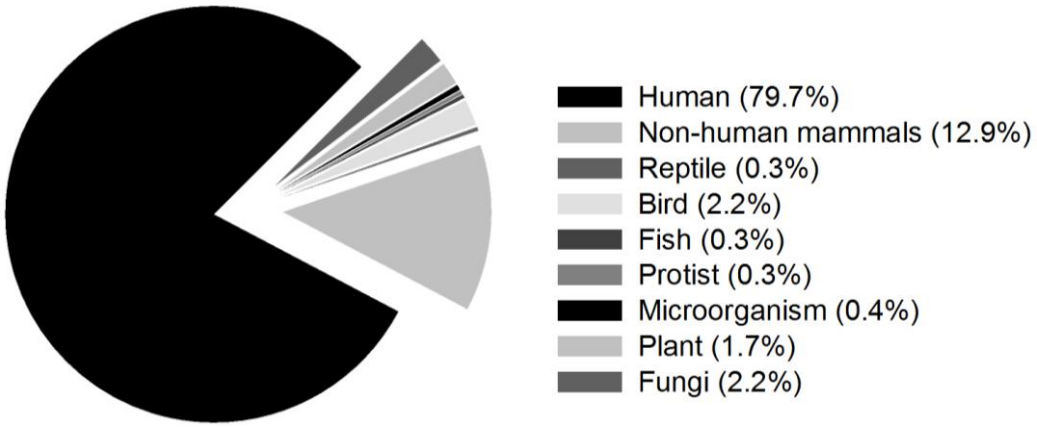

Figure 4. Summary of the taxonomic focus of published papers between 1987 and 2008 identified by the Web of Science literature search.

Clearly, there is a paucity of telomeric research on teleosts (bony fish) and chondrichthyans (sharks, skates, rays, and chimeras). What research that has been conducted on fish (teleosts and chondrichthyans) has been directed at characterising the telomeric sequence (e.g. Salvadori et al. 1995; Rocco et al. 1996; Fontana et al. 1998; Garrido-Ramos et al. 1998; Gornung et al. 1998; Perez et al. 1999; Rocco et al. 2001; Chew et al. 2002).

Only recently have the relationships between telomere length and chronological age been investigated in teleosts – with conflicting findings (Table 1). In total, seven teleost species have been examined to date, with significant correlations between telomere length and chronological age having been identified in the mangrove snapper (*Lutjanus argentimaculatus* Forsskal) (Tsui 2005), the bluefin killifish (*Nothobranchius rachovii* Ahl) (Hsu et al. 2008), and the medaka (*Oryzuas latipesi* Temminck & Schlegel)

(Hatakeyama et al. 2008). Alternatively, significant telomere length and age relations were not found in the black sea bream (*Acanthopagrus schlegeli* Bleeker) (Tsui 2005), the European sea bass (*Dicentrarchus labrax* Linnaeus) (Horn et al. 2008), and the zebra fish (*Danio rerio* Hamilton-Buchanan) (Lund et al. 2009). Interestingly, a more recent paper showed that two laboratory strains of the same species of turquoise killifish (*Nothobranchius furzeri* Jubb) showed contrasting patterns of telomere length change with age (Hartmann et al. 2009).

Table 1. Comparisons of teleost telomere length-at-age regression parameters. n/a: data not available. State: (×) no significant change in telomere length with age; and (✓) telomere length declines with age.

Species	r^2	*P*-value	State	Reference
Acanthopagrus schlegeli	0.21	0.059	×	Tsui (2005)
Danio rerio	n/a	n/a	×	Lund et al. (2009)
Dicentrarchus labrax	0.03	> 0.05	×	Horn et al. (2008)
Lutjanus argentimaculatus	0.15	< 0.05	✓	Tsui (2005)
Nothobranchius rachovii	n/a	< 0.001	✓	Hsu et al. (2008)
Nothobranchius furzeri (MZM-0403 strain)	n/a	0.004	✓	Hartmann et al. (2009)
Nothobranchius furzeri (GRZ strain)	n/a	> 0.05	×	Hartmann et al. (2009)
Oryzias latipes	0.33	0.010	✓	Hatakeyama et al. (2008)

These findings imply that patterns of telomere length change with chronological age are not a shared trait among teleost species and in the unusual case of the *N. furzeri* vary within laboratory strains of a single species (Table 1). Thus broader taxonomic sampling is required to better understand the variable nature of patterns of telomere length change with age within this diverse taxonomic group. Furthermore, to date there have been no investigations into relations between telomere length and chronological age within the chondrichthyes and the marine mammals, constituting a significant knowledge gap.

Moreover, these studies highlight that the different teleost species examined to date show variable rates of telomere length change with age. This inter-specific variability is commonly seen in other taxonomic groups (Haussmann et al. 2003; Vleck et al. 2003; Nakagawa et al. 2004; Tsui 2005). The rate at which telomere lengths change over a species lifespan has been shown to be an important correlate of species longevity

(Monaghan & Haussmann 2006). In birds and mammals, the rate of telomere length change with chronological age is correlated inversely with longevity (Haussmann et al. 2003; Vleck et al. 2003). For example, humans lose telomeres at a rate of 55 bp yr^{-1} and may attain maximum longevities in excess of 100 years; while the telomeres of mice shorten at a rapid rate of 600 bp yr^{-1} throughout their 3 to 4 year life spans (Coviello-McLaughlin & Prowse 1997; Feng et al. 1999; Haussmann et al. 2003). However, due largely to the limited data set available for the fishes, these patterns have not been assessed.

Globally there are an estimated 30,000 plus species of extant fish (Froese & Pauly 2008). As a taxonomic group, the fishes are unique in the breadth of diversity of their life history patterns and longevities (Bone et al. 1995). Longevities of fish can exceed 100 years, with the longest-lived species being the rockeye roughfish (*Sebastes aleutianus* Jordan & Everman) reaching a staggering 205 years of age (Cailliet et al. 2001). This is in stark contrast to some genera of the killifishes (Order Cyprinodontiformes), which are represented by "annual" species, i.e. longevities < one year (Bone et al. 1995; Valdesalici & Cellerino 2003).

Identifying those mechanisms which contribute to the observed variation in longevity among species is fundamental in understanding inter-specific variability in rates of reproduction and mortality (Rose 1991; Mangel & Abrahams 2001); as well as providing an insight into the evolution of life history strategies (Stearns 1992; Haussmann & Mauck 2008). However, the factors that define these remain largely unknown in fishes (Cailliet et al. 2001; Mangel & Abrahams 2001) and require further investigation as a greater understanding of the mechanisms that contribute to intra-specific variability of rates of telomere length change in fishes may elucidate the factors that moderate species" longevities.

Variable patterns of telomere length change with age

As early as 1990, inverse relationships between telomere length and chronological age were observed in *in vivo* and *in vitro* mammalian research (Hastie et al. 1990), which initially led to the paradigmatic view of telomeres undergoing declines in length with the chronological age of the donor. More recently, several papers have documented the

relationships between telomere length and chronological age in a range of other jawed vertebrate (gnathostome) species (refer to Table A1, Appendix One).

Of the 777 research papers from the Web of Science literature search, only 197 (25.4 %), reported some form of interaction between animal/donor age and telomere length. Of these 9.1 % (n = 18) specifically targeted telomeres as an age determinate. When the statistical power of these 197 papers reporting some form of interaction between animal/donor age and telomere length was assessed, over half (54.8 %) of reported interactions between telomere length and age were significant, i.e. probability values (P–values) < 0.05. However, many papers that reported non-significant telomere length-at-age interactions did not detail the P–values of their analysis, potentially underestimating the prevalence of age-independent telomere length change.

This expansion of the taxonomic groups studied within the gnathostomes has resulted in the description of three different states of telomere length change with chronological age. The first, traditional and more common pattern of telomere length change is an overall decline in telomere length with age. This pattern is best characterised by the mammals; however, this has not been assessed adequately in marine mammals. The second more uncommon pattern is an increase of telomere length with age, which has only been reported for a single bird species, Leach‟s storm-petrel (*Oceanodroma leucorhoa* Vieillot) (Haussmann et al. 2003). Finally, the third pattern is an absence of significant telomere length change with age, which is characteristic of the turtles for instance (Girondiot & Garcia 1998; Hatase et al. 2008).

These studies have shown that by expanding the taxonomic focus of telomeric research, the once generalised view of telomere length decline with chronological age is not universally shared among all gnathostome lineages and that paradigms based on mammalian telomere biology require testing in a wider array of gnathostomes in order to establish the taxonomic generality.

Evolutionary aspects of telomere length variability

The description of three different patterns of telomere length change with chronological age within the gnathostomes requires further understanding of the biological, molecular

and evolutionary factors that selectively constrain species to a particular pattern. Thus three important questions require addressing:

- What are the selective factors that constrain species in having one pattern of telomere length change with age over an alternate pattern?
- What are the selective factors that drive transitions between patterns of telomere length change with age between taxonomic units (e.g. orders, families, or species)?
- What are the benefits and, or consequences of a species possessing one of these three variable patterns of telomere length change with age as opposed to an alternate pattern?

In order to adequately address these questions, we first need to identify the overall evolutionary distribution of patterns of telomere length change in the gnathostomes. This will enable future research to target taxonomic groups with recent histories of transitions between patterns of telomere length change with age. To date, information outlining the distribution of patterns of telomere length change with age among the gnathostomes is limited to a few advanced teleosts and otherwise to the tetrapod vertebrates (Fig. 4: refer to Table A1, Appendix One). Patterns of telomere length change with age are not shared amongst all lineages of tetrapods, for example the African clawed frog (*Xenopus laevis* Daudin), the only amphibian to be investigated so far, does not show a relationship between telomere length and age whereas, almost all mammalian vertebrates do (Bassham et al. 1998). The available literature indicates that the teleosts do not share a singular pattern of telomere length change with age (Table 1). In addition, as there have been no investigations into the relationship between telomere length and age in the chondrichthyes and the marine mammals, determining the evolutionary origin(s) of patterns of telomere length change are severely impeded.

This thesis

I aimed to assess the application of telomere length measurements as an age determinate for free-living populations of aquatic vertebrates – chondrichthyans (sharks, rays, skates, and chimaeras) [Chapter Two], teleosts (bony fishes) [Chapter Three], and pinnipeds (seals, sea lions, and walruses) [Chapter Four]. I also compared the performance of three commonly used methods for measuring telomere lengths [Chapter

Two]. In meeting these objectives, I significantly expanded the current taxonomic sampling of telomere length-at-age relations in fishes (teleosts and chondrichthyans).

This expansion of coverage of jawed vertebrate telomere length relationships with age should enhance our understanding of the distribution of patterns of telomere length change with age among the jawed vertebrates and further our understanding of the correlative role of telomeres in animal aging [Chapter Five]. Moreover, this taxonomic expansion of telomere length-at-age relations will provide a better understanding of where the evolutionary origin(s) of patterns of telomere change lie and can be used to direct future research into the causes and consequences of transitions between, or the acquisition of these patterns [Chapter Six].

The specific aims of this thesis were to:

- Evaluate the application of measurements of telomere length as a novel means of determining the ages of free-living chondrichthyans, teleosts, and pinnipeds.
- Assess patterns of telomere length change with chronological age in multiple chondrichthyan species, the first such survey.
- Significantly expand the taxonomic range of osteichthyan fishes examined for correlations between telomere length and chronological age, with the inclusion of the ceratodontiform species, the Australian lungfish (*Neoceratodus forsteri* Krefft); the sister group to the tetrapod vertebrates.
- Test whether the pattern of declining telomere lengths as seen in terrestrial mammals, is shared with marine mammals, by comparing telomere lengths between age classes of a pinniped species, the Australian sea lion (*Neophoca cinerea* Péron).
- Test for correlations between variable rates of telomere length change with age and longevity among species of chondrichthyans and teleosts.
- Finally, to identify the overall evolutionary patterns of transitions between states of telomere length change with age in the jawed vertebrates.

“Ever seen a shark with wrinkles?”

…Sara Filoche (2005)

Chapter Two

Telomere length varies independently of age in the Port Jackson shark, *Heterodontus portusjacksoni*: with a commentary on telomere length measurement methods

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Text in manuscript

Statement of Authorship

In this chapter, Christopher Izzo collected the specimens, performed the qPCR and TRF assays and increment ageing, analysed the data and wrote the manuscript. Terry Bertozzi had input in the development of the experimental protocols and the manuscript. Bronwyn Gillanders and Stephen Donnellan assisted in the synthesis of the manuscript.

Certification that the statement of contribution is accurate.

Christopher Izzo (Candidate)

Signed *Date* 07/01/2010

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Terry Bertozzi

Signed *Date* 07/01/2010

Stephen C. Donnellan

Signed *Date* 07/01/2010

Bronwyn M. Gillanders

Signed *Date* 07/01/2010

Chapter Two Preface

Chapter Abstract

Telomere length change has been suggested as a method for determining the ages of free-living animals, but this prospect has not been examined in the cartilaginous fishes, i.e. sharks, rays, skates and chimaeras. We examine the relationship between telomere length and age in the Port Jackson shark (*Heterodontus portusjacksoni*), a readily obtained exemplar of the sharks. Tissues from Port Jackson sharks ranging in age from 0 to 17 years were sampled from the gulf region of South Australia. Telomere lengths were estimated in four types of tissues, using three measurement methods, both the relative and absolute quantitative PCR (qPCR) assays as well as the terminal restriction fragment (TRF) analysis. We did not find any relationship between telomere length and age in any of the four tissue types, using any method, indicating that telomeres are not suitable ageing structures for this species. Telomere lengths measured by each method were significantly but weakly correlated, as a result of differences in the performance of both classes of measurement techniques. We review the various methodological limitations that influence the accuracy of measures of telomere length for both classes of techniques and suggest methodological improvements and the adoption of better technology that may result in improved accuracy.

Chapter Acknowledgments

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Chapter Two

Telomere length varies independently of age in the Port Jackson shark, *Heterodontus portusjacksoni*: with a commentary on telomere length measurement methods

Introduction

For more than 80 years, counts of growth increments in the calcified structures (i.e. vertebrae and fin spines) of chondrichthyans (sharks, rays, skates and chimaeras) have been the only means of determining the ages of free-living individuals (Cailliet 1990). However, these methods are limited by calcified structure availability, the subjectivity of increment patterning and interpretation, and destructive sampling (Cailliet & Goldman 2004). Therefore, the development of alternative ageing methods for chondrichthyans is essential.

Telomeres are highly conserved oligonucleotide repeats (TTAGGG), found at the ends of vertebrate chromosomes (Moyzis et al. 1988) that stabilize chromosomes and prevent DNA degradation and fusion events (Blackburn 1991; Saldanha et al. 2003). In a wide range of animal species telomere length shortens with age and these changes are a useful age determinate in some species of mammals, birds, and reptiles (Haussmann & Vleck 2002; Nakagawa et al. 2004; Scott et al. 2006). More recently, telomere length has been shown to shorten with age in teleost fishes (Tsui 2005; Hatakeyama et al. 2008; Hartmann et al. 2009). However for chondrichthyans, aside from limited studies of the nucleotide sequence composition of their telomeres (Rocco et al. 2001), nothing is known of the relationship between telomere length and age in this biologically diverse and ecologically important group of marine vertebrates.

The terminal restriction fragment (TRF) analysis has been the most widely used method for telomere length measurement (Harley et al. 1990; Nakagawa et al. 2004; Thomas et al. 2008). It is an estimate of the mean length of terminal restriction fragments per chromosome. However, the TRF method requires large amounts of DNA (0.5 to 5 μg

per individual) and a substantial time investment for analysis (3 to 5 days) (Baird 2005; Lin & Yan 2005). Fluorescence *in situ* hybridization (FISH) is also used commonly to measure telomere length (McNeil & Ried 2000; Lin & Yan 2005), but FISH-based methods are technically and logistically difficult for many wildlife species because of the requirement for metaphase chromosomes; they are also expensive and time consuming (Callicot & Womack 2006).

Cawthon (2002) introduced the measurement of telomere length by the real-time quantitative polymerase chain reaction (qPCR), which uses precisely designed telomere sequence specific PCR primers that avoid amplifying primer-dimer derived products. Using qPCR the relative quantity of the telomeric repeat unit is determined by comparing the quantity of telomere repeat product to the quantity of a single copy gene product; thus, the resulting ratio of telomere to single copy gene quantity is proportional to the average telomere length (Cawthon 2002). Quantitative PCR requires picograms of DNA and large numbers of samples can be analysed in a short space of time when compared to non-PCR based techniques (Cawthon 2002; Gil & Coetzer 2004; Callicott & Womack 2006). However, the first generation qPCR-based method was limited in that it only provided a relative measure of telomere length, which required calibration with a TRF analysis (Baird 2005; O'Callaghan et al. 2008). O'Callaghan et al. (2008) overcame this key limitation of the conventional qPCR based method by including a PCR of a synthetic oligomer telomeric standard to generate absolute telomere length per diploid genome rather than a relative quantification of telomere length.

Here we assess variation in telomere length with chronological age in the Port Jackson shark (*Heterodontus portusjacksoni* Meyer). The Port Jackson shark is an easily accessed species for assessing telomere ageing, in that it is encountered commonly throughout its range and a wide representation of age classes are obtained readily. Moreover, it is handled easily and is harmless. We assessed telomere length with both the conventional relative and absolute qPCR assays as well as the terminal restriction fragment (TRF) analysis, allowing for a direct comparison of the relative advantages and limitations of these measurement techniques. Whilst our study focuses predominately on the use of skeletal muscle biopsies, we investigated a range of tissues that vary in mitotic activity as potential targets for telomere ageing. Our study is the first to investigate the application of telomere length as a determinate of age in

chondrichthyans, with these findings providing a basis for telomere based ageing for cartilaginous fishes in general.

Methods

Specimen collection

During 2006 and 2007, 40 specimens of *H. portusjacksoni* from a range of sizes were obtained from commercial fishers operating in Spencer Gulf and Gulf St Vincent in South Australia. Immediately following capture, blood was drawn from the caudal vein and specimens were euthanased and stored frozen whole. Individual specimens were later thawed in the laboratory and multiple tissues were taken: skeletal muscle and gonad biopsies and caudal fin clippings. Sharks were sexed based on external morphology (i.e. presence or absence of claspers), and total body length (±mm) and body weight (±g) were recorded.

Assigning maturity condition and age determination

Males of *H. portusjacksoni* were considered sexually mature when the claspers were rigid, the vas deferens showed partial or complete coiling and semen was present (Tovar-Ávila et al. 2007). Females were considered mature when oocytes in the ovaries were > 3 mm in diameter, and if the uteri were differentiated from the oviducts and the oviducal glands were heart shaped (Tovar-Ávila et al. 2007).

In order to calibrate telomere length measurements to animal age, vertebrae were collected from the *H. portusjacksoni* specimens and counts of growth increments were made following standard protocols (Cailliet & Goldman 2004). Increment formation in the vertebrae of Port Jackson sharks has been shown to occur annually; therefore counts of increments provide an accurate measure of animal age (Tovar-Ávila et al. 2008). Vertebral centra were cleaned of excess tissue and then embedded in a clear epoxy resin. Sections (~300 µm) were cut using a lapidary saw. These sections were then mounted onto a microscope slide and examined under a dissecting microscope with a transmitted light source. Counts of increments were made from two calcified structures from each specimen without prior knowledge of the size, sex or previous count of the specimen. If the counts varied between the two centra, a third vertebral centra was prepared and examined. If the third count matched either of the previous two it was

taken as the consensus increment number. If there was no agreement between any of the three counts then that specimen was excluded from the analysis.

DNA extraction

DNA was isolated from blood and tissue samples following prescribed methods (PUREGENE DNA Isolation Kit: Gentra Systems). Sample concentrations were quantified with a plate spectrophotometer and DNA quality was assessed by agarose gel electrophoresis.

In order to test the utility of the telomere primers originally designed for use in mammals, we performed standard PCR assays in a range of chondrichthyan species and a teleost species. Tissues from additional fish specimens and mouse controls were obtained from the South Australian Museum.

Absolute qPCR assay

In order to calculate the relative quantity of the telomeric unit, the quantity of telomere repeat product was compared to the quantity of a single copy gene product (Cawthon 2002). We used the recombination activation gene: *RAG-1*, which has been shown to be a single copy gene in elasmobranches (Schluter & Marchalonis 2003; López et al. 2006).

All PCR primers were evaluated and optimised for the qPCR assays using standard PCR techniques and gel electrophoresis. Annealing temperatures were optimized by running thermal gradient cycles. Each PCR was carried out in a volume of 15 μl with a final concentration of 1 × GENEAMP PCR Gold buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer and 0.5 U of AMPLITAQ GOLD DNA polymerase (Applied Biosystems). All standard PCR assays were performed on a Gradient Palm-Cycler (Corbett Life Science). Amplifications consisted of an initial denaturation step of 94 °C for 9 min; followed by 34 cycles of PCR with the following temperature profile: denaturation at 94 °C for 45 sec, annealing at 62 °C (telomere reactions) or 58 °C (*RAG-1* reactions) for 45 sec, and extension at 72 °C for 60 sec, with an additional final extension at 72 °C for 6 min. Forward and reverse telomeric primers were 5′ CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT 3′ and 5′ GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT 3′ respectively (Callicott &

Womack 2006). Forward and reverse primers for the *RAG-1* were F – 5′ ACC TAC ATA AGA CTT TGG CTC A 3′ and R – 5′ GAT TCA TTC CCT TCA CTT G 3′, respectively.

All quantitative PCR assays consisted of 10 µl reactions that included 5 µl 2 × EXPRESS SYBR GREENER qPCR master mix (Invitrogen), 500 nM ROX reference dye (Invitrogen), 200 mM each of the forward and reverse primers, and approximately 100 ng genomic DNA and were performed in the Rotor-Gene 6000 automated thermocycler (Corbett Life Science). Amplifications consisted of an initial denaturation step of 95 °C for 5 min; followed by 40 cycles of PCR with the following temperature profile: denaturation at 95 °C for 10 sec and annealing at 62 °C (telomere reactions) or 58 °C (*RAG-1* reactions) for 20 sec; followed by an extension and data acquisition step at 72 °C for 15 sec. Melt curves (ramping from 55 – 95 °C) were run at the end of all qPCR reactions in order to identify primer-dimer product formation.

The number of oligomer repeats in each oligomer standard was calculated following O'Callaghan et al. (2008). Briefly, the molecular weights (MW) of the telomere oligomer and the *RAG-1* PCR product standards were calculated. The MW of each standard was divided by Avogadro's number to determine the weight of one molecule of oligomer. The number of copies of the respective telomere and *RAG-1* oligomer standards was calculated by dividing the oligomer standard weight by the weight of one molecule. This value equated to the number of telomeric repeat units in the reaction. Telomere length (in kb) was calculated by multiplying the number of molecules of oligomer by 84 (the telomeric oligomer length). This value also equates to the number of copies of the single copy gene (*RAG-1*) products in the reaction, which is halved (as there are two copies of *RAG-1* per diploid genome), giving the numbers of copies per diploid genome.

Standard curves for the telomeric repeat and the single copy gene *RAG-1* were calculated using the Rotor-Gene 6000 analytical software (Corbett Life Science). The standard curve for telomeric repeats was calculated using serial dilutions of known quantities of a synthesized 84–mer oligonucleotide containing only TTAGGG repeats (GeneWorks) in order to measure the content of telomeric sequence per sample, which could then be related to telomere length in kb (O'Callaghan et al. 2008). The *RAG-1*

standard curve was calculated by serially diluting known concentrations of the *RAG-1* amplification product generated by standard PCR modified by running it for 80 cycles to produce large amounts of the *RAG-1* target sequence. *RAG-1* PCR products were cleaned using a vacuum plate clean-up procedure and their concentration determined by electrophoresing a specified volume against a ladder of a range of molecular weights (MassRuler Low Range DNA Ladder: Fermentas International).

Triplicate assays were run for all paired telomere and the *RAG-1* reactions and the respective telomere lengths. *Relative* measures of telomere length, i.e. the ratio of telomere repeat copy number to the *RAG-1* copy number, were calculated following Cawthon (2002). In order to determine *absolute* telomere lengths, the numbers of *RAG-1* copies per reaction were calculated by relating the C_T values to the standard curves. Telomere lengths per reaction were divided by the numbers of *RAG-1* copies per diploid genome, to give a final telomere length in kb per diploid genome (O"Callaghan et al. 2008).

Terminal restriction fragment analysis

Terminal restriction fragment (TRF) length measurement was performed using the TeloTAGGG Telomere Length Assay (Roche Diagnosis). Approximately 1 µg μl^{-1} of DNA was digested with the restriction enzymes *Hinf*I and *Rsa*I for 2 h at 37 °C. The digested DNA was separated by gel electrophoresis at 5 V cm^{-1} in a 1 % w v^{-1} agarose gel in 1 × TAE buffer.

DNA, transferred to a positively charged nylon membrane by Southern blotting, was hybrdigoxigenin (DIG)-labelled probe specific for telomeric repeats and incubated with a DIG-specific antibody bound to an alkaline phosphate. The telomere probe was visualised through incubation in alkaline phosphatase metabolising CDP-Star, then exposed to X-ray film, which was scanned with the analytical software Quantity One (Bio-Rad). The resulting images were enhanced for clarity with the Quantity One software using a *whole-image background subtraction* tool to improve the contrast of the banding patterns and the image was filtered of excess noise in using the *filter wizard* function.

Using the method described by Tsuji et al. (2002), average telomere lengths were calculated by comparing the telomeric signal relative to a molecular weight standard supplied with the Roche kit. The Quantity One software was able to calculate the regression curve used to estimate the values of the unknown telomeric bands. Those telomeric bands that exceeded 21 kb (the largest weight marker of the kit) were estimated based on the Quantity One derived regression curve. Duplicate measures of TRF (telomere) length were run for all samples.

Statistical analysis

The linear relationships between telomere length measurements from the various tissues and specimen age were assessed by regression analysis. Analysis of variance (ANOVA) was used to compare the telomere lengths between males and females and between immature and mature specimens of *H. portusjacksoni*. ANOVA was used to assess whether telomere length differed among different tissues, and a Bonferroni post-hoc analysis was used to determine which tissues differed when significant differences were found. Regression analysis compared telomere lengths derived by the TRF and qPCR measurement techniques. Statistical analyses were performed using SPSS 15.0 software.

The variability and precision of repeated measures of telomere lengths were analyzed for both the qPCR and TRF techniques using the coefficient of variation (CV) and index of precision (D), which are defined as:

$$\mathrm{CV}_j = 100 \times \frac{\sqrt{\sum_{i-1}^{R} \frac{(X_{ij} - X_j)}{R-1}}}{X_j} \ ,$$

$$\mathrm{D}_j = \frac{\mathrm{CV}_j}{\sqrt{R}}$$

Where, X_{ij} is the *i*th measurement of the *j*th shark, X_j is the mean telomere length of the *j*th shark, and R is the number replicates (Chang 1982).

Results

Utility of the telomeric PCR primers

Agarose gel electrophoresis of PCR products confirmed that the telomere primers were suitable for use in non-mammalian taxa, as the resulting smears were consistent with the rodent positive control (Fig. 1). No PCR products were formed when template genomic DNA was omitted from the reaction, confirming the absence of primer-dimer products (Fig. 1).

Standard curves

The slopes of the standard curves were used to calculate the efficiency of the reactions (Sigma Aldrich 2008). The slopes of the standard curves generated were –3.3 and –3.5 for the telomere and *RAG-1* reactions, respectively, which was within the accepted efficiency thresholds (–3.6 > slope > –3.1) and equates to a reaction efficiency of 100 % (r^2 = 0.99) and 93 % (r^2 = 0.98) for the telomere and *RAG-1* reactions, respectively, as calculated by the Rotor-Gene 6000 analytical software (Corbett Life Science) (Fig. 2).

Quantitative PCR assay

In total, 33 *H. portusjacksoni* specimens (20 females and 13 males) varying in total body length (from 193 mm to 870 mm) were analysed for telomere length in muscle tissue using qPCR. Specimens ranged from 0 to 17 years based on vertebral increment counts. Both measures of relative and absolute telomere lengths in muscle tissue were not significantly different between male and female *H. portusjacksoni* specimens (ANOVA: $P > 0.05$), and did not differ between maturity states (19 immature and 14 mature) (ANOVA: $P > 0.05$).

Relative telomere lengths of muscles were constricted in relation to animal age and showed a trend of minor telomeric attrition over time (Fig. 2A). Relative telomere length were not significantly related to age ($n = 33$, $r^2 = 0.197$, $P = 0.256$) (Fig. 3A), nor total body length ($n = 33$, $r^2 = 0.243$, $P = 0.159$).

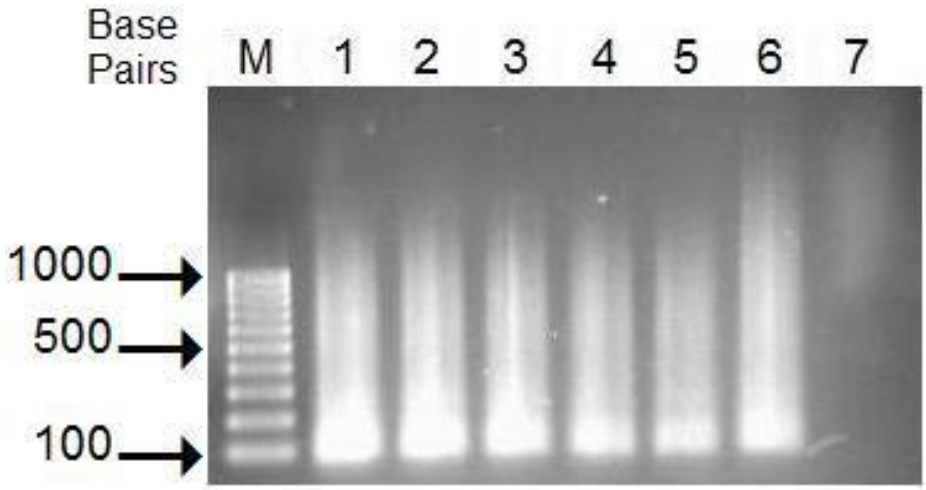

Figure 1. Agarose gel electrophoresis of telomeric PCR products from representative specimens of a: (1) rodent (*Mus musculus* Linnaeus) positive control; (2) shark (*Heterodontus portusjacksoni*); (3) ray (*Trygonorrhina dumerilii* Castelnau); (4) skate (*Spiniraja whitleyi* Iredale); (5) chimaera (*Callorhinchus milii* Bory de Saint-Vincent); (6) teleost (*Macquaria ambigua* Richardson); and (7) no template control. M – is the molecular weight marker in base pairs.

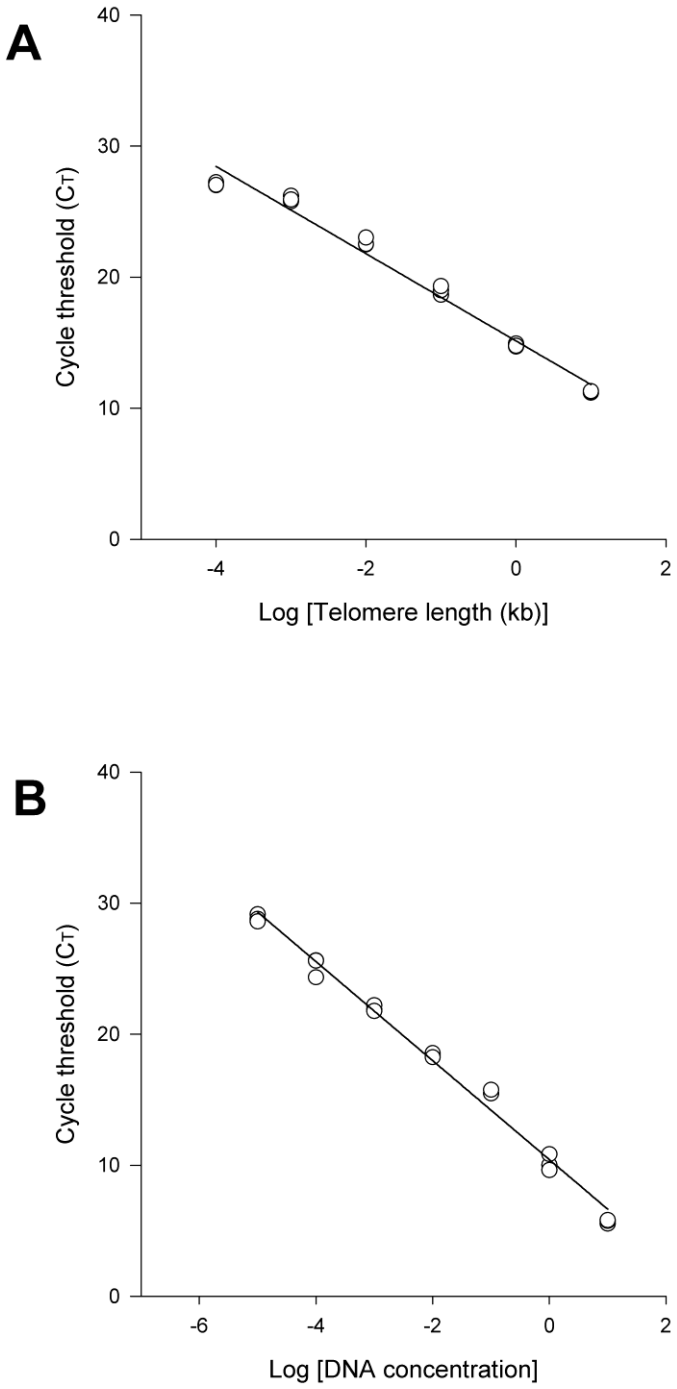

Figure 2. Standards curves used to calculate relative DNA concentrations for qPCR. Where cycle threshold (C_T) is the number of PCR cycles required to generate enough SYBR GREEN fluorescence above the background signal. Standard curves were generated by the Corbett Rotor-Gene software package. (A) Standard curve for calculating telomere lengths ($r^2 = 0.98$). (B) Standard curve for calculating *RAG-1* copy numbers ($r^2 = 0.99$).

Absolute telomere lengths in muscle appeared to be highly variable between specimens and throughout the life span of *H. portusjacksoni,* varying from 85.9 kb per diploid genome to 590.1 kb per diploid genome (Fig. 2B). Interestingly, the individual with the largest measured absolute telomeres was a young of the year shark possessing clear signs of umbilical healing, indicating that it was newly hatched. Absolute telomere lengths in muscle showed a trend towards decreasing length with age; however, no significant relationships were identified between absolute telomere length and the ages of the specimens ($n = 33$, $r^2 = 0.227$, $P = 0.063$) (Fig. 2B). Absolute telomere lengths were also not significantly related to total body length ($n = 33$, $r^2 = 0.277$, $P = 0.052$).

In five young (0 to 1 year of age) and five old individuals (10 to 17 years of age), telomere lengths were measured from fin clippings, gonad biopsies and blood samples. Both the relative and absolute qPCR measurement techniques showed no significant relationships between telomere length and age were identified in fin clippings ($P > 0.05$), gonad biopsies ($P > 0.05$) and blood samples ($P > 0.05$). Measures of telomere length were significantly different between all tissues (ANOVA: $P < 0.001$), with Bonferroni post-hoc analysis identifying fin clippings as having significantly larger telomeres.

Terminal restriction fragment analysis

For measures of mean telomere length by TRF analysis, 34 specimens of *H. portusjacksoni* (19 females and 15 males) ranging in total body length from 225 mm to 870 mm were examined. Based on increment counts these specimens ranged from 0 to 17 years of age. Mean telomere lengths in muscle were not significantly different between sexes (ANOVA: $F_{2, 32} = 1.675$, $P = 0.182$), and maturity states (20 immature and 14 mature) (ANOVA: $F_{2, 32} = 0.391$, $P = 0.536$).

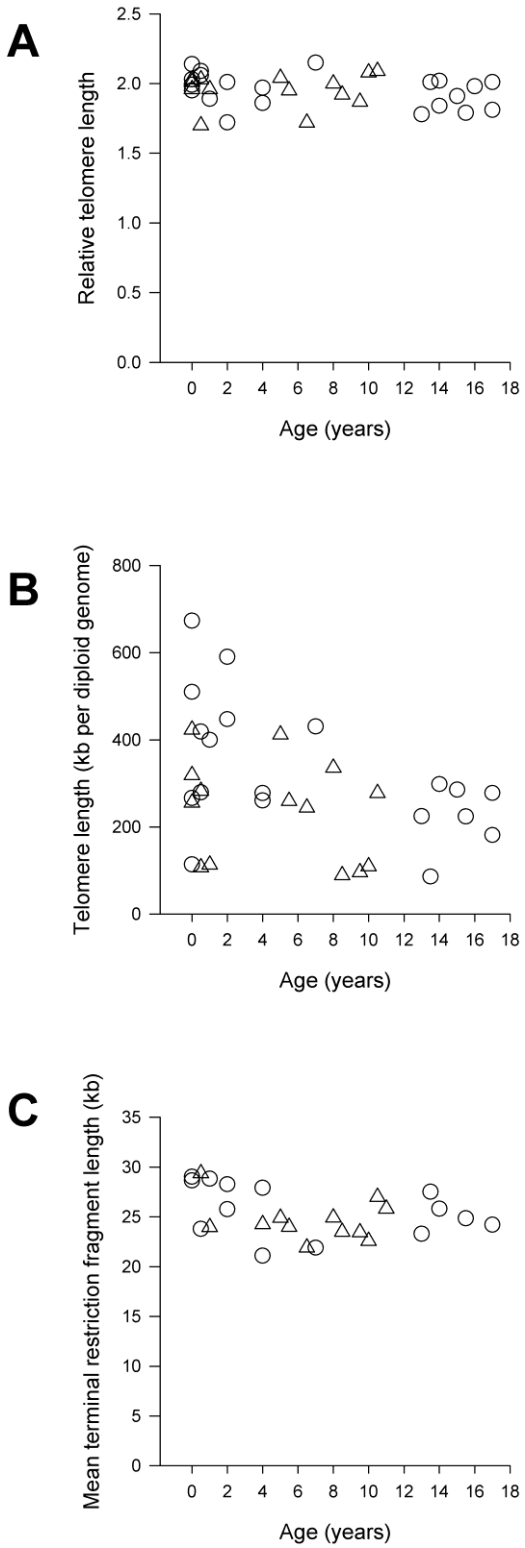

Figure 3. Telomere lengths as estimated by: (A) relative quantitative PCR; (B) absolute quantitative PCR; and (C) terminal restriction fragment analysis, as a function of age in the Port Jackson shark (*Heterodontus portusjacksoni*), where: △ – males, and ○ – females.

Mean telomere lengths in muscle measured by the TRF assay were less variable than those measured by absolute qPCR and showed a pattern of minimal telomere length loss akin to that of the relative qPCR method (Fig. 2A – C). TRF derived mean telomere lengths in muscle varied from 21.10 kb to 29.86 kb (Fig. 2C). Again, while mean telomere lengths declined with animal age (Fig. 2A), mean telomere length and specimen age were not related significantly ($n = 34$, $r^2 = 0.229$, $P = 0.103$) and neither were mean telomere length and total body length ($n = 35$, $r^2 = 0.247$, $P = 0.123$).

We compared measures of mean telomere lengths from five young (0 years of age) and five old individuals (14 to 17 years of age), in fin clippings, gonad biopsies and blood samples. No significant relationships between mean telomere length and age were identified in fin clippings ($P = 0.075$), gonad biopsies ($P = 0.457$) and blood samples ($P = 0.714$). Measures of mean telomere length were significantly different between all tissues (ANOVA, $F_{3, 60} = 4.401$, $P = 0.007$).

Comparison of methods

For logistical reasons, only 23 of the total 40 specimens of *H. portusjacksoni* were analysed for telomere lengths from skeletal muscle tissue using the TRF assay and both the PCR measurement techniques. All telomere length measurements were log transformed to normalise the distribution for each variable.

There was a significant correlation between the relative and absolute telomere lengths ($n = 33$, $r^2 = 0.564$, $P = 0.045$) (Fig. 4A). Regression analysis identified a significant relationship between the relative qPCR and TRF telomere length measurements ($n = 23$, $r^2 = 0.409$, $P = 0.050$) (Fig. 4B). Similarly, a significant relationship was identified between the absolute qPCR and TRF telomere length measurements ($n = 23$, $r^2 = 0.494$, $P = 0.016$) (Fig. 4C).

The mean coefficient of variation (CV) for the relative qPCR method was 2.4 % and the mean measure of the precision (D) of repeated measurements of telomere length was 1.7 %. When the data were transformed into absolute telomere lengths, the measures of error (CV and D) increased to 6.6 % and 3.8 %, respectively. These were less than the mean CV and D of repeated TRF derived mean telomere length analyses, which were 8.2 % and 5.8 %, respectively.

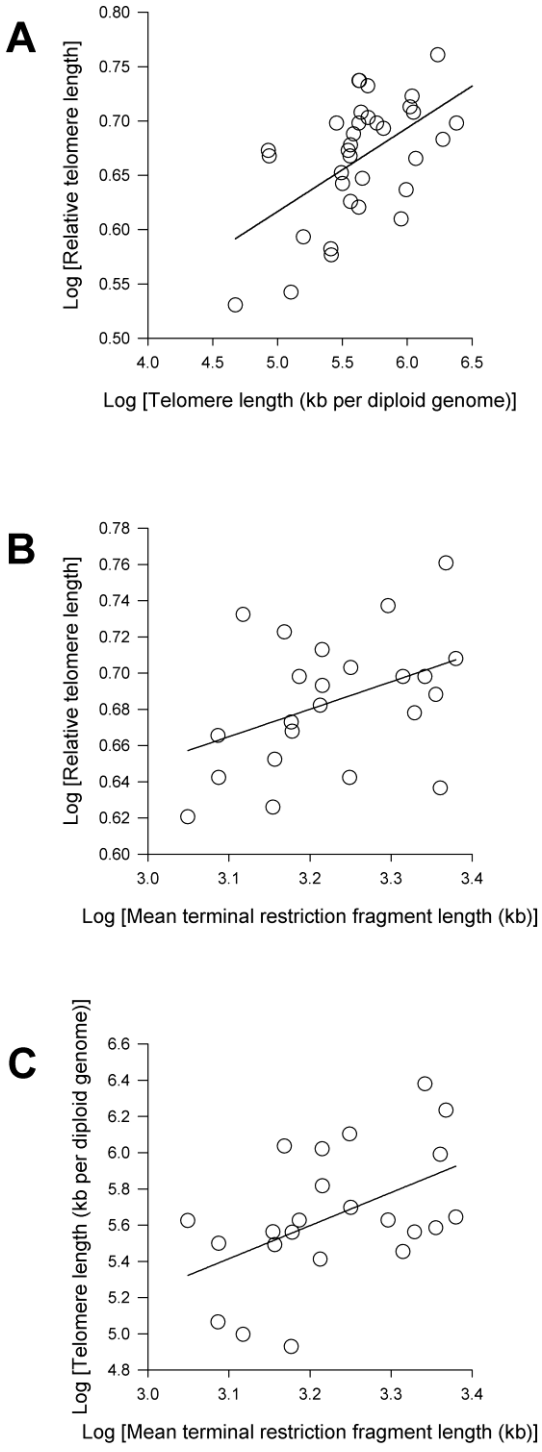

Figure 4. Comparison of two methods for measuring the lengths of telomeres in muscle biopsies from the Port Jackson shark (*Heterodontus portusjacksoni*): (A) relative qPCR versus absolute qPCR; (B) relative qPCR versus terminal restriction fragment analysis; and (C) absolute qPCR versus terminal restriction fragment analysis. The solid line is the line of best fit.

Discussion

Relationship of telomere length with age

Using three methods of telomere length measurement, we found that there was no significant relationship between relative, absolute or mean telomere length and animal age in muscle tissues of *H. portusjacksoni*. While muscle tissue has been used previously to successfully identify relationships between telomere length and age in a number of teleost species (Tsui 2005; Hatakeyama et al. 2008; Hartmann et al. 2009), muscle is considered a relatively mitotically inactive tissue and as such, telomere lengths may remain stable over time (Monaghan & Haussmann 2006; Aviv 2008). However, the examination of more mitotically active tissues also failed to show significant telomere length-at-age relationships. Our finding indicates that telomeres may not be suitable generally as an age determinate in chondrichthyan fishes. Furthermore, these findings also indicate that telomeric sequence loss is not related directly to tissue turnover rates. Indeed other stochastic factors potentially act upon telomere attrition rates during aging, i.e. chronic disease (Djojosubroto et al. 2003), and measures of telomere lengths in cross-sectional population studies may reflect differences in initial telomere lengths (Takubo et al. 2002), which themselves are influenced by environmental cues (Hall et al. 2004).

Taxonomic utility of qPCR telomere length estimates

We have shown that the telomere specific primers originally designed for use in mammals successfully amplify the telomeric repeat unit in all fish surveyed, indicating that the qPCR assays have the potential to be applied in measuring telomere lengths in all aquatic vertebrates. This is not surprising as the telomeric sequence is highly conserved among vertebrate groups (Moyzis et al. 1988). The application of qPCR to other chondrichthyans (and other vertebrate species in general) is not limited by the availability of an appropriate single copy gene. The *RAG-1* gene is ubiquitously present in vertebrates, single copy in diploid species, and its widespread use for molecular clock dating means that nucleotide sequences are available for a large number of vertebrate groups (e.g. Hugall et al. 2007). Furthermore, the standardization to a uniform single copy gene for future qPCR studies may enhance the validity of inter-specific comparisons of telomere size by qPCR (Criscuolo et al. 2009).

Telomere length measurements

The range of TRF derived mean telomere lengths for *H. portusjacksoni* was 21 to 29 kb, which is within the range of values reported for vertebrates – mammals (9 to 22 kb), birds (9 to 30 kb), reptiles (16 to 34 kb), and teleosts (3 to 25 kb) (Haussmann et al. 2003; Nakagawa et al. 2004; Tsui 2005; Scott et al. 2006; Bronikowski 2008; Hatakeyama et al. 2008; Horn et al. 2008; Criscuolo et al. 2009; Lund et al. 2009; Ujvari & Madsen 2009).

For the TRF analysis we used constant field gel electrophoresis (CFGE) to separate the telomeric fragments. However, the upper size limit of DNA fragments that CFGE can separate is approximately 30 kb, i.e. the limit of mobility (Burmeister & Ulanovsky 1992; Southern & Elder 1995). While many of our telomere lengths were within a size range of 21 to 29 kb, there is the possibility that the larger telomeric smears may have been compressed; thus, underestimating the upper length range. Avoiding the limit of mobility of CFGE can be achieved through the use of pulse field gel electrophoresis (PFGE), which is able to separate large DNA fragments up to 10 Mb (Haussmann & Mauck 2008). Haussmann and Mauck (2008) compared the performance of the two electrophoretic methods on the correlation of telomere length and age, and while PFGE showed a stronger relationship, the relationship determined from CFGE was still significant, indicating that CFGE can be an acceptable alternative when PFGE equipment is not available.

Absolute telomere length estimates from the qPCR approach can be converted to per chromosome telomere lengths, the same parameter produced by the TRF approach (O¨Callaghan et al. 2008; Thomas et al. 2008), by dividing the absolute telomere length (in kb per diploid genome) by twice the chromosome number (i.e. the number of telomeres in the genome). While the chromosome number of *H. portusjacksoni* has not been determined, two congeners have chromosome numbers of 102 (Schwartz & Maddock 2002). If we assume the same diploid chromosome number in *H. portusjacksoni*, then the mean telomere length per chromosome from the qPCR method ranges from 0.42 to 2.89 kb. This estimate would be reduced once the contribution of telomeric sequences from interstitial and pericentromeric regions (so-called ITS of some authors) was taken into account. Clearly the qPCR and TRF estimates of mean telomere length per chromosome are not equivalent, differing by an order of magnitude

at least. A similar but smaller disparity is found for these methods with human white blood cell telomere length estimates (TRF range: 6.3 to 11.3 kb: Okuda et al. 2002; Benetos et al. 2005, and the qPCR range: 1.1 to 1.5 kb, our calculations on data from Thomas et al. 2008). Presently an explanation for this disparity (presumably due solely to the qPCR estimate) is not apparent. However, despite this disparity, a relationship with age and the qPCR estimate of telomere length is still apparent in humans.

Methodological considerations

Our three measures of telomere length were significantly but weakly correlated to one another. We expected there to be a stronger correlation than observed based on comparisons of the methods in humans ($r^2 = 0.88$, $P < 0.0001$: Thomas et al. 2008) and two species of birds ($r^2 = 0.87$, $P < 0.001$ and $r^2 = 0.76$, $P < 0.005$: Criscuolo et al. 2009). Both the TRF and qPCR measurement techniques are subject to artefacts, which affect the accuracy of telomere length measurements (Aviv 2008). The TRF method is vulnerable to subjectivity and random errors, whereas the qPCR method, while less subjective, has potentially more sources of random errors. We now assess the impact of these sources of error on each method.

- *TRF errors*

The TRF assay provides a mean of the distribution of telomere lengths across all of the chromosomes (Saldanha et al. 2003), under the assumption that the distribution of telomere lengths is normal. If not, the mean telomere length may be under- or over-estimated as a result of a non-Gaussian distribution of telomere lengths (Aviv 2008). Two potential causes for a non-Gaussian distribution can be identified, one biological in origin and the second methodological. First, the presence of telomeric sequences from interstitial and pericentromeric locations could also influence the intensity of the telomeric smears at the low and high molecular weight regions respectively. The influence of interstitial telomeric sequences on the TRF method may be negligible, as these are generally quite short in mammals at least, i.e. < 100 bp (Ruiz-Herrera et al. 2008). Selection of regions of the telomere smear that are not influenced by the presence of telomeric sequences from interstitial or pericentromeric locations for analysis may alleviate this problem (e.g. Haussmann & Mauck 2008). Second, the possible compression of fragments at the higher molecular weight end of telomere

smears in CFGE may be a contributor to the low correlation between TRF and qPCR derived telomere lengths.

In the TRF assay, the sub-telomeric region located between the restriction enzyme cutting site and the proximal end of the telomere sequence is included in the measurements (Baird 2005). The sub-telomeric region varies between individuals (up to 5 %) and can further amplify the amount of variation in telomere lengths between individuals (Levy et al. 1992; Cawthon 2002; Mefford & Trask 2002).

The interpretation of the hybridisation smears on the autoradiographic images of the TRF assay is somewhat subjective, and may be hampered by image clarity and telomeric smear contrast issues (Joula et al. 2006; Haussmann & Mauck 2008). Aside from image quality issues, detailed analyses of the information content of regions of the telomere smear show that objective selection of particular regions markedly improves accuracy (Haussmann & Mauck 2008). However, the application of such approaches is dependent on the presence of a correlation of telomere length change and age, and in those species where this relationship does not exist; such an optimisation approach would not be possible. In the latter case, we suggest that the lengths of the smallest telomeres could be reported, as the smaller telomeres may be considered the most biologically meaningful given that telomere attrition to critically short lengths arrests normal cellular function (Hemann et al. 2001).

- *qPCR errors*

The relative qPCR method as described by Cawthon (2002) provides a relative measure of telomere length, the T/S ratio, which is equivalent to the factor that the telomere repeat copy number (T) of a DNA sample differs from a single gene copy number (S). The T/S ratio is essentially unit less and can be assigned a base pair value by calibration to samples of known telomere lengths (Cawthon 2002). However, this calibration value may vary between individuals of different ages (Aviv 2008).

The presence of telomeric sequences from interstitial and pericentromeric locations would be an issue for the accuracy of telomere length measurements for the qPCR method (Meyne et al. 1990; Nakagawa et al. 2004). These sequences have been observed in a wide range of vertebrates (Meyne et al. 1990) including some batoids and

teleosts, but not in either of the two galaeomorph sharks examined to date (Rocco et al. 2001). However, the inclusion of these sequences would only be an issue for the accuracy of measurement of telomere length if they are subject to intra-specific variation, otherwise telomere lengths would be over-estimated by a constant factor (Nakagawa et al. 2004), and if they occurred infrequently would contribute little to qPCR length estimates. Currently, no estimates of the number and length of telomeric sequences from interstitial and pericentromeric locations are available for any chondrichthyan.

The qPCR techniques are subject to systematic and random errors from the PCR amplification process that would affect the accuracy and precision of telomere length measurements (Nordgård et al. 2006; Aviv 2008). Slight variations in the efficiency of the PCR reactions, especially if present from the earliest cycles, have the potential to result in a T/S ratio with lowered accuracy. Additionally the standard curves developed from the synthetic telomeric oligomer utilised by the absolute qPCR methodology, may fail to adequately replicate the complexity of a biological template in the telomeric and single copy gene assays. Nordgård et al. (2006) recommend using at least two single copy reference gene assays and performing multiple replicates for standard curves (we performed triplicate replicates for the standard curves) as a means of reducing error propagation from stochastic PCR sources. The biological template complexity issues in test sample PCR remains unresolved.

The values of the coefficient of variation we report for both measurement methods were comparable to those reported for counts of growth increments in fish (Campana 2001). As noted by Aviv (2008) even an extremely conservative error value of 1 % may provide a substantial over-estimate an individual‟s actual age. Hence, there may be a requirement to establish a set telomere length measurement error threshold (i.e. ≤ 5 %). This error may be more of an issue for short-lived species (i.e. the annual cyprinodontiform fishes) than long-lived species (i.e. marine mammals, large sharks), where age estimates with confidence intervals of two to four years are still valuable (Dennis 2006).

Concluding remarks

When high levels of variability in telomere lengths independent of age make telomeres not suitable for estimating actual animal age, or even defining broad age/size classes (Monaghan & Haussmann 2006), telomere length may, nevertheless, provide insights into the life history of individual animals (e.g. Bize et al. 2009). However, the commonly applied measurement techniques are subject to various methodological limitations that influence the accuracy of measures of telomere length. Methodological improvements (e.g. use of multiple reference genes in qPCR, optimal window identification for the TRF method) and the adoption of better technology (e.g. PFGE), should result in improved accuracy. Furthermore, the standardisation of methodological protocols will allow for valid comparisons among studies, e.g. telomere length values reported in the literature may represent either the mean of the distribution of all telomere lengths, or only a sub-set of the distribution, e.g. the smallest or longest telomeres (Aviv 2008).

“I know the human being and fish can coexist peacefully”

…George W. Bush (2000)

Chapter Three

Variation in telomere length of the common carp, *Cyprinus carpio* (Cyprinidae) in relation to age and tissue type

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Statement of Authorship

In this chapter, Christopher Izzo collected the specimens, performed the quantitative PCR assays and animal ageing, analysed the data and wrote the manuscript. Terry Bertozzi had input in the development of the experimental protocols and the manuscript. Bronwyn Gillanders and Stephen Donnellan assisted in the synthesis of the manuscript.

Certification that the statement of contribution is accurate.

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Signed *Date* 07/01/2010

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Chapter Three Preface

Chapter Abstract

Determinations of the age structure of wild teleost populations are vital for sustainable management and conservation efforts. Yet, the commonly applied increment based ageing technique is limited by the subjectivity of increment patterning and interpretation, and destructive sampling. Here we assess the application of telomere length as an alternate age determinate in wild caught specimens of the common carp (*Cyprinus carpio* Linnaeus). Telomere lengths were measured from muscle biopsies and fin clips using the absolute quantitative PCR method and correlated to animal age estimates based on otolith increment counts and length-at-age calculations. Measures of telomere lengths did not differ between sexes; however, state of sexual maturity and tissue type influenced telomere lengths. Measurements of telomere lengths were highly variable in both muscle biopsies and fin clips from the same individual; however, telomeres from muscle biopsies significantly increased in length at a rate of 13.8 kilo bases per diploid genome per year. These findings conflict with the more generalised pattern of telomere length attrition, marking the second reported case of significant telomere elongation with age in vertebrates. Overall, these findings indicate that telomere length does not provide a suitable means of determining age, or assigning broad age cohorts based on measurements of length. Alternatively, telomeres may be better suited as indices of animal condition, by providing a measure of the "physiological" age of individuals, which reflect the accumulated effects of stress events throughout the lives of individuals.

Chapter Acknowledgments

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Chapter Three

Variation in telomere length of the common carp, *Cyprinus carpio* (Cyprinidae) in relation to age and tissue type

Introduction

Estimates of fish age, upon which calculations of rates of growth and mortality are based, are the most informative life history characteristic for managing fish populations (Campana & Thorrold 2001). The determination of fish age is generally based on the enumeration of growth increments in the calcified structures of fish: otoliths, scales, vertebrae, as well as fin spines and rays (Casselman 1983). Whilst commonly used as age determinates, these structures are generally limited by the subjectivity of increment patterning and interpretation, availability, and the general requirement to destroy the specimen (Metcalf & Swearer 2005).

Previous studies have shown that in some teleost species, telomere lengths change predictably over time (Tsui 2005; Hatakeyama et al. 2008; Hartmann et al. 2009), and have the potential to provide a novel and non-lethal fish ageing technique for free-living populations. Telomeres are repeated nucleotide sequences located at the ends of eukaryotic chromosomes (Blackburn 1991). Telomeres function to protect the chromosome from DNA damage and subsequently undergo processes that cause changes in the lengths of the telomeres (Blackburn 1991). Telomere lengths are also influenced by extrinsic factors, such as stress, both perceived and physiological (i.e. oxidative damage) (von Zglinicki et al. 2000; Epel et al. 2004). Yet, most studies on non-mammalian taxa do not assess the influence that sex and state of sexual maturity have on telomere lengths, two biological parameters that are generally acknowledged as affecting the growth of teleosts (Nikolskii 1969). Furthermore, the assessment of telomere lengths in teleosts have utilised relatively short-lived species that were captive reared and thus potentially do not provide an accurate representation of telomere length dynamics over an extended period of time under natural conditions.

The common carp (*Cyprinus carpio* Linnaeus) provides a suitable model teleost species for telomere ageing studies. Native to Eastern Europe and central Asia, *C. carpio* has since been translocated throughout the world, becoming a highly successful invader of freshwater habitats (Koehn 2004). In Australian freshwater systems, *C. carpio* is abundant and readily available from the wild (Koehn 2004) allowing for easy sampling. Furthermore, the annual periodicity of growth increment formation in the otoliths of *C. carpio* has been previously validated (Vilizzi & Walker 1999; Brown et al. 2004), showing that counts of increments provide accurate estimates of age.

Here we use the common carp as a model teleost species to assess variation in telomere length in relation to a wider range of biological variables than have been examined previously in fishes. The development of a non-lethal teleost ageing protocol based on the measurement of telomere lengths will be invaluable for the management and conservation of exploited and endangered fish stocks. We aim to outline the precise nature of telomere length-at-age relations in wild caught *C. carpio*. Two tissues (muscle biopsies and fin clips) were examined in order to compare the rate of change of telomere length between tissues from the same specimen. Furthermore, the effects of sex and state of sexual maturity (i.e. mature versus immature) on telomere lengths were assessed.

Methods

Sample collection and DNA extraction

Specimens of *C. carpio* were obtained from the River Murray, South Australia between November 2008 and March 2009 and from the Torrens River, South Australia in early 2009. Once caught, specimens were euthanased and stored whole on ice or frozen. Individual specimens were later processed in the laboratory, where they were sexed through macroscopic examination of the gonads (ovaries and testes) and maturity was assessed. When gonads lacked development (i.e. not able to distinguish sex), the fish was classified as juvenile (immature). Finally, fork length (FL: ± mm) and body weight (total weight minus gonad weight; BW: ± g) were recorded. Where possible, otolith pairs were also removed in order to determine the chronological age of the specimen.

Muscle was biopsied from the ventral body wall and caudal fin clips were collected. Both tissues were then stored frozen or in ethanol prior to DNA extraction. Due to their

small size, larval fish were used whole for DNA extractions. DNA was isolated following prescribed methods (Gentra Systems). After extraction, DNA quality was assessed through gel electrophoresis.

Primer optimisation

PCR conditions for primer pairs were optimised using standard PCR techniques and gel electrophoresis. Annealing temperatures were optimized by running thermal gradient cycles. Each PCR was carried out in a volume of 15 μl with a final concentration of 1 × GENEAMP PCR Gold buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer and 0.5 U of AMPLITAQ GOLD DNA polymerase (Applied Biosystems). All standard PCR assays were performed on a Gradient Palm-Cycler (Corbett Life Science). Amplifications comprised an initial denaturation step of 94 °C for 9 min, followed by 34 cycles with the following temperature profile: denaturation at 94 °C for 45 sec, annealing gradient from 52 – 64 °C for 45 sec, and extension at 72 °C for 60 sec, with an additional final extension at 72 °C for 6 min. The optimal annealing temperature for both primer pairs was 62 °C.

Forward and reverse telomeric primers were from Callicott and Womack (2006). The recombination activation gene (*RAG-1*) was used to determine the relative quantity of the telomeric repeat sequence (Cawthon 2002). Sequences of forward and reverse *RAG-1* primers were: F – 5′ ATC ACC AAC TAC CTC CAC AAG AC 3′ and R – 5′ CCC AGA CTC ATT GCC TTC AC 3′, respectively.

Absolute quantitative PCR

Absolute quantitative polymerase chain reactions (qPCR) for both the telomere and *RAG-1* assays were carried out in a volume of 10 μl with a final concentration of 1 × EXPRESS SYBR GREENER qPCR master mix (Invitrogen), 500 nM ROX reference dye (Invitrogen), 200 mM each of the forward and reverse primers, and approximately 100 ng genomic DNA. All qPCR assays were performed in the Rotor-Gene 6000 automated thermocycler (Corbett Life Science). Amplifications comprised an initial denaturation step of 95 °C for 5 min, followed by 40 cycles with the following temperature profile: denaturation at 95 °C for 10 sec and annealing at 62 °C for 20 sec, followed by an extension and data acquisition step at 72 °C for 15 sec. Melt curves (ramping from 55 to 95 °C) were run at the end of all qPCR reactions.

Standard curves

The standard curve for telomeric repeats was calculated using serial dilutions of known quantities of a synthesized oligonucleotide consisting of 14 repeats of the telomeric sequence (O'Callaghan et al. 2008). The *RAG-1* standard curve was calculated by serially diluting known concentrations of the *RAG-1* amplification product of the standard PCR. In order to relate the cycle threshold (C_T) values to telomere lengths, we calculated the number of telomeric oligomer repeats and *RAG-1* templates in each standard following O'Callaghan et al. (2008). Standard curves and reaction efficiencies for the telomeric repeat and the single copy gene *RAG-1* assays were calculated using the Rotor-Gene 6000 analytical software (Corbett Life Science). The resultant slopes of the standard curves were –3.3 ($r^2 = 0.98$) and –3.4 ($r^2 = 0.99$) for the telomere and *RAG-1* reactions, respectively, which equates to a reaction efficiency of 100 % and 96 % for the telomere and *RAG-1* reactions, respectively.

For each specimen assessed, triplicate assays were run for both telomere and the *RAG-1* reactions. The resultant C_T values were then related to the respective standard curves in order to determine the telomere lengths and numbers of *RAG-1* copies per reaction. Telomere lengths were then divided by the numbers of *RAG-1* copies per diploid genome, to give a final telomere length in kilo base pairs (kb) per diploid genome.

Determination of specimen age

In order to calibrate telomere length measurements to chronological age, counts of growth increments in the sagittal otoliths were made. Otoliths were embedded in a clear setting epoxy resin and approximately 300 µm sections were cut using a lapidary saw. Two counts of growth increments were made from an otolith from each specimen without prior knowledge of the size, sex or previous count of the specimen under a dissecting microscope with transmitted light. If the two counts varied, a third count was made. If the third count matched either of the previous two it was taken as the consensus increment number.

Where otoliths or consensus increment counts were not available, specimen age was calculated by fitting fork length data to sex specific length-at-age functions, described by Vilizzi and Walker (1999). These length-at-age functions are based on validated age

data from the same river system, and hence, are assumed to provide an accurate estimate of animal age.

Statistical analysis

The linear relations between telomere length and specimen age were assessed by regression analysis. Analysis of variance (ANOVA) was used to compare the telomere lengths between sexes and maturity states, where mean telomere length was the dependent variable and the factors were sex and maturity. An ANOVA was used to assess differences in telomere lengths between the different age cohorts. Statistical analyses were performed using SPSS 15.0 software.

Results

In total, 84 specimens of *C. carpio* (44 females, 35 males and 5 larvae of unknown sex) ranging in FL from 201 to 639 mm (excluding the larval fish which ranged in length from 4 to 8 mm) were analysed for telomere lengths. Specimens ranged in age from 0 to 9 years based on otolith increment counts and length-at-age calculations. In the wild *C. carpio* generally attains an age of up to 11 years (Brown et al. 2003); therefore, our study provides a good range of specimen ages to assess telomere lengths throughout the life history of the species.

In general, females possessed longer telomeres than males. However, telomere lengths were not significantly different between sexes for muscle (ANOVA: $F_{1, 78} = 0.017$, $P > 0.05$), or fin clips (ANOVA: $F_{1, 78} = 1.156$, $P > 0.05$), thus telomere length-at-age data were combined for sexes for further analysis (Fig. 1).

To assess the effects of state of sexual maturity on telomere lengths, specimens of *C. carpio* were divided into mature ($n = 72$) and immature ($n = 12$). A significant difference in telomere lengths was identified between the maturity states for muscle (ANOVA: $F_{1, 83} = 4.867$, $P < 0.05$), but not in fin clips (ANOVA: $F_{1, 83} = 0.064$, $P > 0.05$) (Fig. 2).

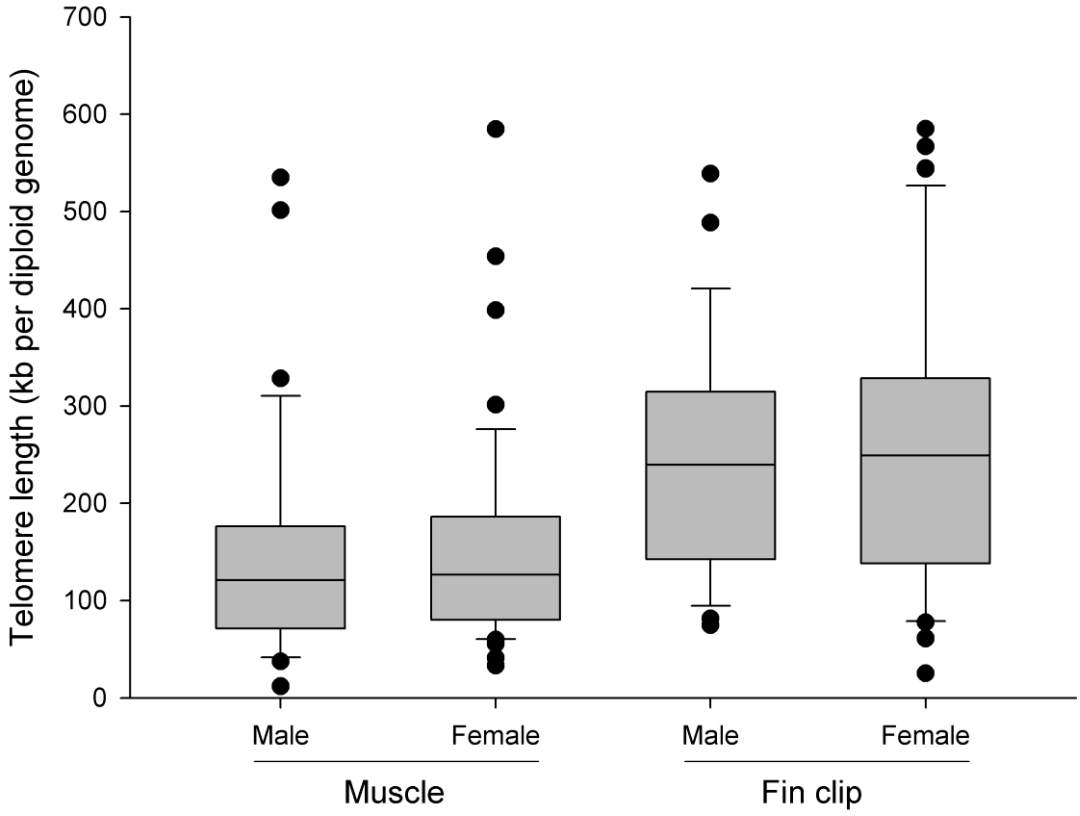

Figure 1. Telomere lengths measured in male and female specimens of the common carp (*Cyprinus carpio*) from muscle biopsies and fin clippings. The boxes represent the ranges of telomere lengths measured. The mean is shown by the horizontal line in the boxes and the tails are ± the standard errors of the mean. Where ● = outlying values.

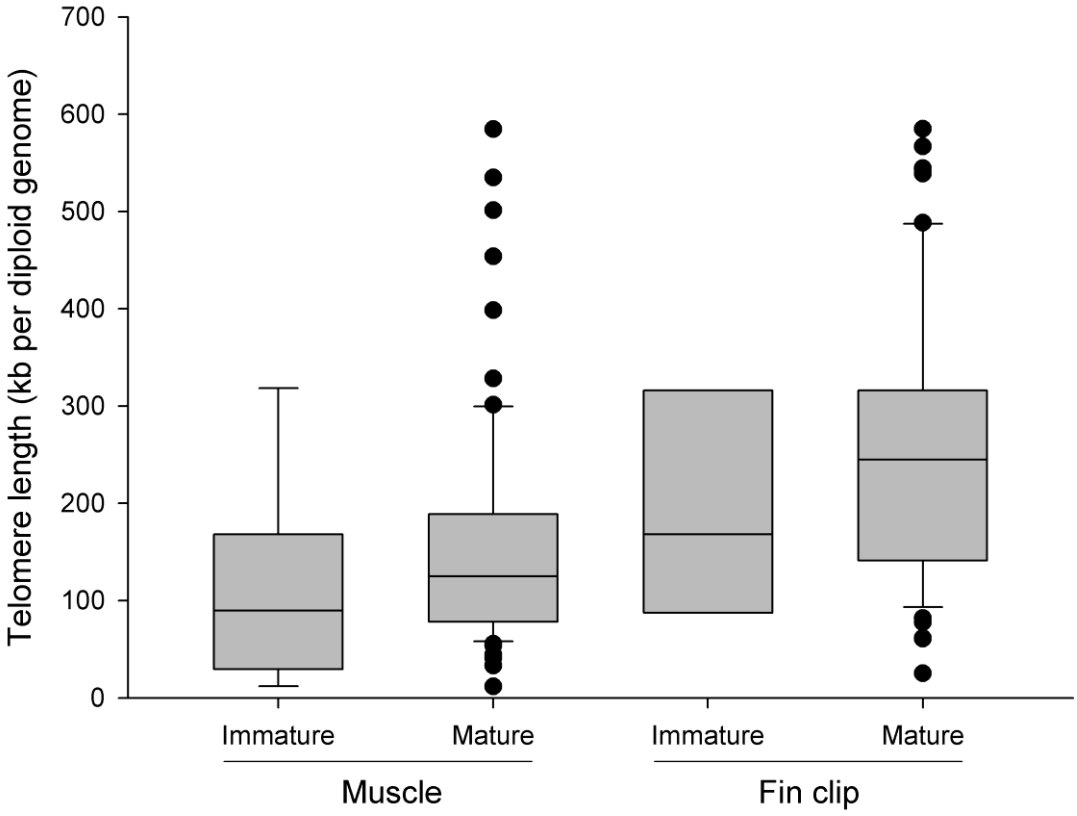

Figure 2. Telomere lengths measured in immature and mature specimens of the common carp (*Cyprinus carpio*) from muscle biopsies and fin clippings. The boxes represent the ranges of telomere lengths measured. The mean is shown by the horizontal line in the boxes and the tails are ± the standard errors of the mean. Where ● = outlying values.

As DNA was isolated from the whole larval samples and reflects a combination of telomere lengths from multiple tissue types, these telomere lengths were included in both the muscle and fin clip data sets and represented the young of the year cohort (YOY; i.e. 0 years old). Telomeres from fin clips showed no discernable change in length with increasing animal age and telomere length-at-age relations were not significant (linear regression: $n = 84$, $r^2 < 0.0001$, $t = -0.069$, $P > 0.05$) (Fig. 3). The exclusion of larval samples did not improve telomere length-at-age relations in fin clips (linear regression: $n = 79$, $r^2 = 0.010$, $t = 0.863$, $P > 0.05$).

Telomere length measurements from muscle biopsies showed a trend of increasing telomere length with chronological age (Fig. 4). Muscle telomere lengths were significantly related to chronological age (linear regression: $n = 84$, $r^2 = 0.097$, $t = 2.969$, $P < 0.05$). Telomere lengths in muscle tissues increased at a rate of 13.8 kb per diploid genome per year. When larval telomere length measurements were excluded from this data set, telomere length-at-age relations improved marginally based on the correlation coefficient (linear regression: $n = 79$, $r^2 = 0.137$, $t = 3.492$, $P < 0.001$). Telomere lengths in muscle biopsies were significantly related to specimen fork length (linear regression: $n = 84$, $r^2 = 0.054$, $t = 2.154$, $P < 0.05$), and body weight (linear regression: $n = 84$, $r^2 = 0.104$, $t = 3.092$, $P < 0.05$).

The initial telomere length (mean telomere length at birth, i.e. the *Y*–intercept of the regression equation) was 87.43 kb per diploid genome. This was approximately half the mean measured telomere length, 160.9 kb per diploid genome, of the larval samples.

Overall there was a high level of variability in telomere length measurements between and within age cohorts (Fig. 4). Telomere lengths ranged from 11.76 to 584.69 kb per diploid genome. Both of these individuals were 6 year old individuals, and hence this cohort had the largest range in measured telomere lengths. There were no significant differences in the mean telomere lengths of the different age cohorts (ANOVA: $F_{9, 74} = 1.668$, $P > 0.05$).

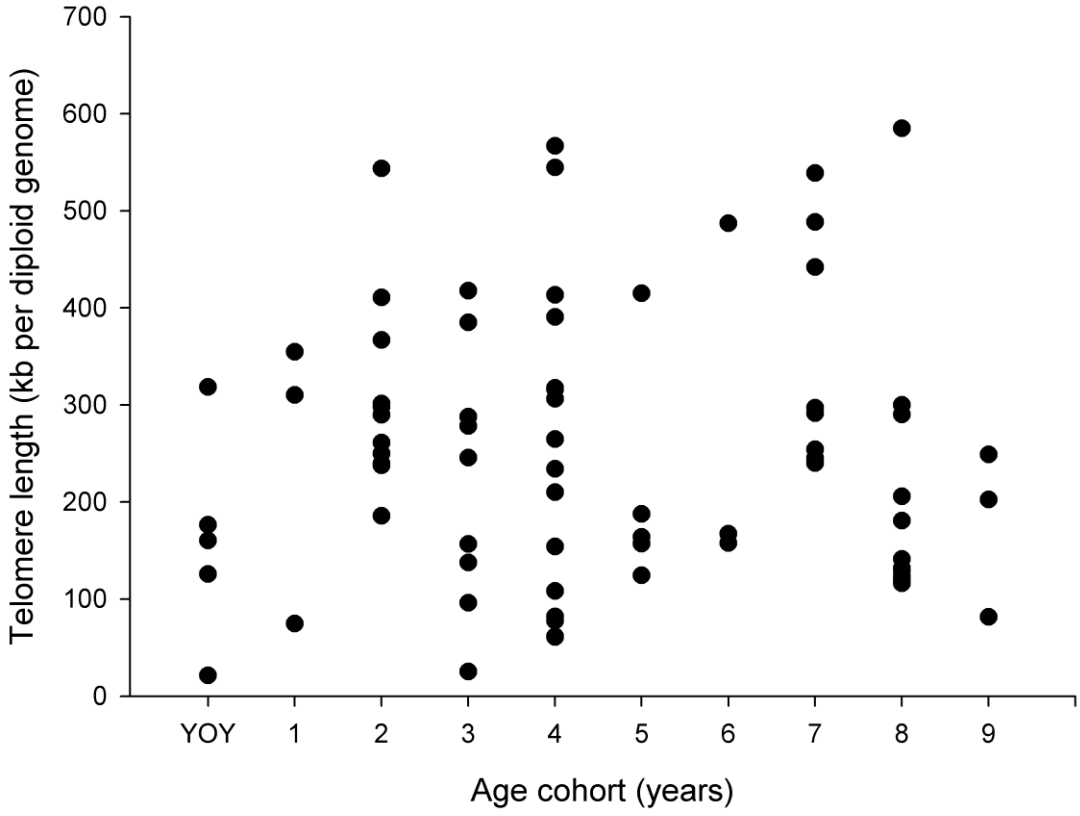

Figure 3. Chronological age of the common carp (*Cyprinus carpio*) as a function of telomere length from fin clippings. Where ● = telomere length measurements of individual fish for combined sexes.

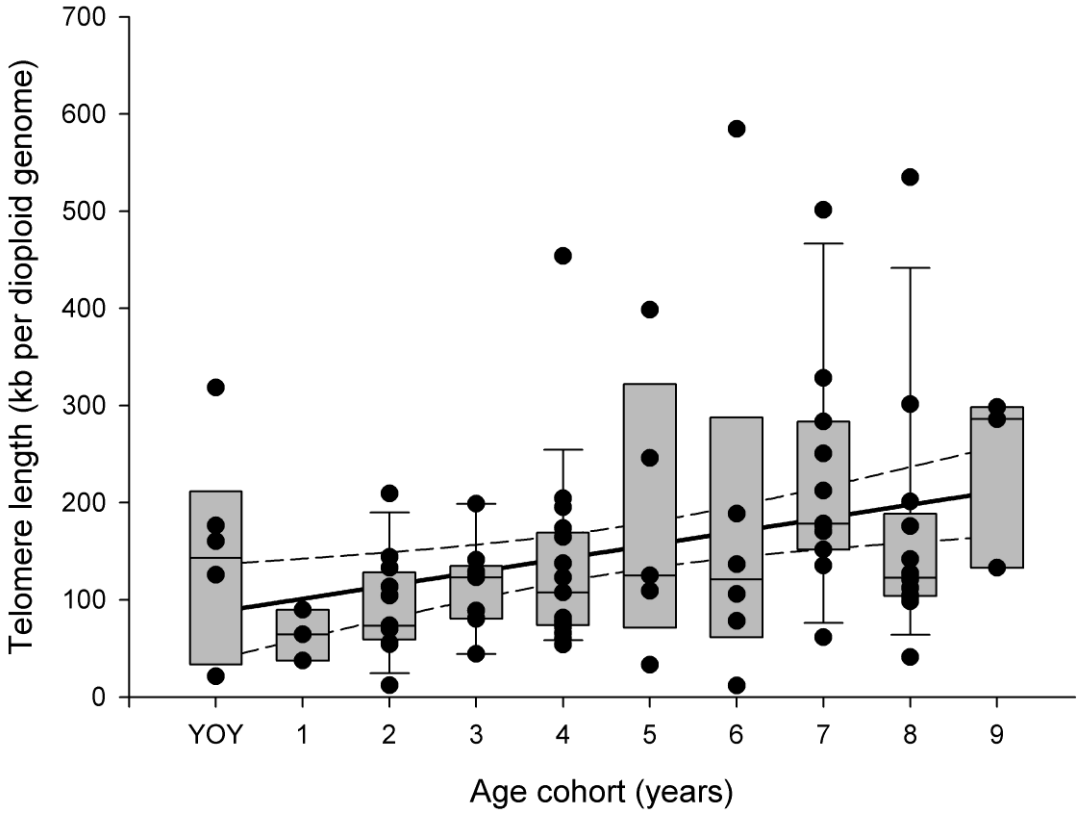

Figure 4. Chronological age of the common carp (*Cyprinus carpio*) as a function of telomere length from muscle biopsies. Where ● = telomere length measurements of individual fish for combined sexes. The solid line is the best fit regression through the data for combined sexes and the dashed lines are the 95 % confidence intervals. The boxes represent the ranges of telomere lengths measured. The mean is shown by the horizontal line in the boxes and the tails are ± the standard errors of the mean.

Discussion

We identified significant telomere length-at-age relations from muscle biopsies taken from wild caught specimens of *C. carpio*. However, significant telomere length-at-age relations were only evident in muscle biopsies and not in fin clip derived telomeres. Similarly, in the Japanese medaka (*Oryzias latipes* Temminck & Schlegel), significant relations between telomere length and age were identified in five of seven tissues assayed (Hatakeyama et al. 2008). Significant telomere length-at-age relations were identified in both mitotically active (i.e. kidney and intestine) and inactive tissues (i.e. muscle), suggesting that tissue surveys are required in order to identify a suitable target tissue for telomere based fish ageing (Nakagawa et al. 2004).

Due to the variation within an age class found in this study, we conclude that the telomere based fish ageing method does not provide the required level of accuracy for determining the age of fish. Previously, high levels of variability in telomere length measurements within the age cohorts of free-living animals led to the suggestion that telomeres are best limited to assigning broad age classes (Haussmann et al. 2003). However, we were not able to differentiate mean telomere lengths between the age cohorts surveyed in this study, implying that telomeres are not even a suitable means of providing an estimate or broad classification of the age of fish. These findings suggest that counts of growth increments in the calcified structures of teleosts will continue to remain the most commonly applied fish ageing methodology. Examination of otoliths and vertebral centra requires destructive sampling of specimens (Metcalf & Swearer 2005), which within a fishery setting may be considered an acceptable practice as part of the monitoring of the age structure of the population. Furthermore, whilst scales and fin spines are generally considered a less reliable ageing structure than otoliths, they potentially provide a non-lethal means of estimating the ages of free-living teleosts (Casselman 1983). The associated variation in counts of growth increments in teleost scales and fin spines is considerably less than that seen here in telomere length measurements (Campana 2001).

One potential explanation for the high variability in telomere length measurements within the age cohorts of the *C. carpio* specimens may be that telomeres more accurately represent the “physiological” age of an individual, i.e. the combined affects of chronological ageing, individual specimen biology and stress (Benetos et al. 2001).

Whilst physiological age may not be analogous to chronological age, it may provide a measure of stress (both physiological and perceived), and act as a bioindicator of animal health and longevity (Cawthon et al. 2003; Juola et al. 2006; Pauliny et al. 2006). Measures of telomere lengths in individuals may also provide a biomarker of environmental stress, either through the exposure of animals to high levels of pollutants, through the loss of suitable habitat, or shifts in feeding regimes (Horn et al. 2008).

We found that there was a significant trend of increasing telomere lengths with age, with the older age cohorts having longer mean telomere lengths than the younger age cohorts. Telomere lengths increase at a rate of 13.8 kilo bases per diploid genome per year in the muscle tissues of *C. carpio*. Telomere elongation has only been previously identified in one other vertebrate species, Leach‟s storm-petrel (*Oceanodroma leucorhoa* Vieillot) (Haussmann et al. 2003). This increase in telomere length was not shared between the two tissues examined in this study, with telomeres in fin clips showing a weak trend of decreasing length with animal age. These findings are comparable to those reported for the black sea bream (*Acanthopagrus schlegeli* Bleeker), which despite having non-significant telomere length-at-age relations, showed a trend of increasing telomere lengths in blood samples and a decrease in length in muscle derived telomeres (Tsui 2005).

The continual increase in telomere lengths as seen in *C. carpio* may facilitate an increased capacity for cellular replication (Allsopp et al. 1992) and reproductive longevity (Aydos et al. 2005; Hanna et al. 2009). Furthermore, telomere extension with age may be influential in providing *C. carpio* with a general pattern of indeterminate growth; that is, low rates of natural mortality (Brown et al. 2005), extended reproductive longevity and increased fecundity with body size (Smith 2005), and negligible senescence (Kishi et al. 2003).

The elongation of telomere lengths in *C. carpio* may also facilitate this species to be such a globally successful invasive freshwater species, by providing greater buffers for “stress events” and the phenotypic plasticity to colonise both pristine and degraded habitats (Fernándex-Delgado 1990; Koehn 2004; Zambrano et al. 2006). Increasing telomere lengths may allow *C. carpio* to have a rapid growth rate, short generation times, and low rates of natural mortality (Koehn 2004; Brown et al. 2005). Long

telomeres are associated with greater resilience to disease (Cawthon et al. 2003), increased juvenile survival (Hall et al. 2004), and may also assist in overcoming the long-term effects of food scarcity (Jennings et al. 2000; Goyns 2002).

Whilst our study identified significant telomere length-at-age relations in the muscle biopsies of wild caught common carp, the high levels of variability within age cohorts and the inability to differentiate between cohorts implies that telomere length is not a suitable means of determining the ages of fish. Thus, otoliths and other calcified structures of teleosts will continue to remain the commonly applied fish ageing structures. Instead, telomere lengths may be more applicable as a measure or index of the individual condition of an animal, by providing a means of measuring the physiological age of the animal, reflecting the accumulated affects of stress events undergone throughout an individual‟s life time. The direct application of measures of telomeres as condition indices requires further development to fully understand the affects of intrinsic and extrinsic factors on the telomere biology of free-living organisms. Furthermore, there still remains the need for additional investigation into the development of novel non-lethal fish ageing techniques in order to overcome the limitations of the currently applied growth increment based fish ageing methodologies.

“It’s better to have the approximate age of a live whale than the exact age of a dead whale”

… Peter Harrison (2006)

Chapter Four

Telomere length analysis for rapidly determining age in pinnipeds: the endangered Australian sea lion as a case study

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Text in manuscript

Statement of Authorship

In this chapter, Christopher Izzo performed the quantitative PCR assays, analysed the data and wrote the manuscript. Derek Hamer collected the specimens and contributed to the development of the manuscript. Terry Bertozzi had input in the development of the experimental protocols and the manuscript. Bronwyn Gillanders and Stephen Donnellan assisted in the synthesis of the manuscript.

Certification that the statement of contribution is accurate.

Christopher Izzo (Candidate)

Signed *Date* 07/01/2010

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Bronwyn M. Gillanders

Signed *Date* 07/01/2010

Chapter Four Preface

Chapter Abstract

Telomeres are the protective caps at the ends of all chromosomes. As a result of faulty replicative DNA mechanisms, telomeres undergo repeated bouts of sequence loss resulting in the progressive shortening of their lengths. This telomere shortening is negatively correlated with age in terrestrial mammals. We test whether this pattern is shared in marine mammals, by comparing mean telomere lengths between age classes in the pinniped species, the Australian sea lion (*Neophoca cinerea* Péron). Telomere lengths were measured in specimens from three age classes: pups, juveniles, and adults. The mean telomere lengths of the adults were significantly smaller than the juvenile and pup classes. However, we were unable to differentiate between pups and juveniles. Similarly, mean telomere lengths did not differ significantly between the sexes. These findings confirm that the Australian sea lion shares the general pattern of shortening telomere lengths with age as documented in terrestrial mammals. The application of telomere lengths as an age determinate requires considerable development to refine the scale of the age estimates derived, which will require the use of known-aged individuals. Nonetheless, measures of telomere lengths have the potential to become valuable tools in molecular ecology.

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Chapter Four

Telomere length analysis for rapidly determining age in pinnipeds: the endangered Australian sea lion as a case study

Introduction

Telomeres are repeated non-coding nucleotide sequences that cap the terminal ends of all eukaryotic chromosomes (Blackburn 1990). Telomeres function to protect the coding ends of chromosomes by buffering against sequence loss as a result of faulty DNA replicative mechanisms and DNA damage events (Blackburn 1990). Telomere lengths may be maintained or extended by one of several means, i.e. telomerase (a ribonucleoprotein complex) or by the alternative lengthening of telomeres (ALT), which currently are only described in mammalian tumours cells (Reddel 2003). However, in most normal somatic cells of mammals, telomerase is not consistently expressed throughout the life of an individual at levels that facilitate telomere maintenance, resulting in the progressive shortening of telomere lengths (Reddel 2003).

In general, mammals share a single pattern of telomere length attrition throughout their lifespan (Hastie et al. 1990; Allsopp et al. 1992). Moreover, this change in the length of telomeres is negatively correlated with age (Hastie et al. 1990; Vaziri et al. 1993). In humans, measurements of telomere lengths have been proposed as a forensic tool for determining the age of individuals (Tsuji et al. 2002; Lahnert 2005). However, there is a paucity of published literature on patterns of telomere length change with age in marine mammals.

The few studies available for pinnipeds suggest their telomeres would be subject to the pattern of changes in length similar to those reported for terrestrial mammals. Intra-chromosomal telomeric repeats have been observed in many vertebrate species, varying in number and location on individual chromosomes (Meyne et al. 1990; Pagnozzi et al. 2000). Telomeric sequences at the terminal ends of chromosomes only were identified

in the pinniped species, the harbor seal (*Phoca vituline* Linnaeus) (Meyne et al. 1990). The absence of „interstitial telomeric sequences" in *P. vituline* is encouraging, because their presence may confound measures of terminally located telomere lengths (Haussmann & Vleck 2003). In addition, Elmore et al. (2008) found that the Steller sea lion (*Eumetopias jubatus* Schreber) and the Beluga whale (*Delphinapterus leucas* Pallas) had undetectable levels of telomerase in somatic tissues, which is indicative of subsequent change in telomere length. Furthermore, given the phylogenetic relatedness between pinnipeds and terrestrial carnivores (Sato et al. 2006), for which relationships between telomere length and age exist (Nasir et al. 2001; Yazawa et al. 2001; Brümmendorf et al. 2002; McKevitt et al. 2002, 2003), it seems logical for the same pattern to exist in pinnipeds.

The further refinement of the telomere based ageing technique may provide a valuable tool for non-invasive determination of the demographic structure of pinniped populations. Such a tool could have broad applications in wildlife biology and forensics. Telomere lengths may provide an immediately applicable forensic tool to identify broad age classes of beached animal carcasses (Tsuji et al. 2002). Moreover, telomeres may be used to forensically monitor the compliance of commercial pinniped hunter"s within demographically based harvest management systems, i.e. the prohibition of targeting hooded and harp seal pups by the Canadian pinniped fishery (Canadian Department of Fisheries and Oceans [DFO] 2009), where only trace samples are available. Forensic applications of telomere length determination are feasible with the recent development of a quantitative PCR assay (Alonso et al. 2004; Swango et al. 2006). The broader application of this technology to other marine mammal species would be equally as valuable. For example, the further modification of telomere based ageing in cetaceans has the potential to eliminate the necessity of lethal sampling of scientific whaling programmes (Oshumi 1995; Brownell et al. 2000; Nakagawa et al. 2004; Dennis et al. 2006).

We test whether the mammalian pattern of telomeric sequence loss is shared by a pinniped species. We assess telomere length change at the population level in the endangered Australian sea lion, *Neophoca cinerea* (Péron), by comparing mean telomere lengths between broad age classes from a single breeding colony using a quantitative PCR based assay.

Methods

Sample collection and DNA extraction

Biopsy samples were collected opportunistically from Australian sea lions at the Olive Island breeding colony (South Australia) in April 2009, while undertaking fieldwork for a broader, conservation-based study of the species. Animals from both sexes were captured while ashore on the island and assigned to one of three broad life history stages: pup, juvenile and adult. Sex was determined by the presence of external reproductive morphology and age was determined using size, pelage colouration and gross morphology (McIntosh 2007). Due to logistical limitations, biopsy samples from adult males were not obtained.

Animals were restrained for a short period of time using a purpose-built V-net and tissue samples were obtained by removing a small (5 mm^2) biopsy from one of the hind flippers. All procedures were approved by the University of Adelaide Animal Ethics Committee (S-008-2006) and conducted under a South Australian Department for the Environment and Herirage permit (A-24684). Flipper biopsies were stored in ethanol prior to DNA extraction. DNA was isolated following prescribed methods (Gentra Systems) and DNA quality was assessed through gel electrophoresis.

Primer optimisation

PCR conditions for primer pairs were optimised using standard PCR techniques and gel electrophoresis. Forward and reverse telomeric primers were taken from Callicott and Womack (2006). The interphotoreceptor retinoid binding protein (*IRBP*) has been shown to be a single copy gene in mammals (Borst et al. 1989; Sato et al. 2003) and was used to determine the relative quantity of the telomeric repeat sequence (Cawthon 2002). Sequences of the forward and reverse *IRBP* primers were: F – 5' CAC TCA CCA ACC TCA CAC AAG A 3' and R – 5' CCA CAT TGC CCT CCA GAA C 3', respectively.

Annealing temperatures were optimized by running thermal gradient cycles. Each PCR was carried out in a volume of 15 μl with a final concentration of 1 × GENEAMP PCR Gold buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer and 0.5 U of AMPLITAQ GOLD DNA polymerase (Applied Biosystems). All standard PCR assays were performed on a Gradient Palm-Cycler (Corbett Life Science). Amplifications

comprised an initial denaturation step of 94 °C for 9 min, followed by 34 cycles of: denaturation at 94 °C for 45 sec, an annealing gradient from 52 – 64 °C for 45 sec, and a 72 °C extension for 60 sec; with a final 72 °C extension for 6 min. The optimal annealing temperature for the telomere and *IRBP* primer pairs were 62 and 55 °C, respectively.

Absolute quantitative PCR

Absolute quantitative PCR (qPCR) reactions for the telomere and *IRBP* assays were carried out in a volume of 10 µl with a final concentration of 1 × EXPRESS SYBR GREENER qPCR master mix (Invitrogen), 500 nM ROX reference dye (Invitrogen), 200 mM each of the forward and reverse primers, and approximately 100 ng genomic DNA. All qPCR assays were performed in the Rotor-Gene 6000 automated thermocycler (Corbett Life Science). Amplifications comprised an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of: 95 °C denaturation for 10 sec and a 62 °C annealing step for 20 sec; followed by an extension and data acquisition step at 72 °C for 15 sec. At the completion of the reactions, melt curves, increasing from 55 to 95 °C, were run to assess for the formation of primer-dimer products.

A standard curve for the *IRBP* assays was calculated using serial dilutions of known concentrations of *IRBP* amplicon. The telomeric standard curve was calculated using serial dilutions of known concentrations of a synthesized telomeric oligomer (GeneWorks) following O'Callaghan et al. (2008). Standard curves and reaction efficiencies for the telomeric repeat and the *IRBP* assays were calculated using the Rotor-Gene 6000 analytical software (Corbett Life Science). The resultant efficiencies of the telomere and *IRBP* reactions were 100 %, indicating specific primer binding and the exponential amplification of the target region with each cycle.

All telomeric and IRBP assays were run in triplicate. In order to convert the resulting cycle threshold (C_T) values into telomere lengths, the number of telomeric repeats and *IRBP* copies per reaction were calculated following O'Callaghan et al. (2008). Telomeric repeat numbers were then divided by the numbers of *IRBP* copies per diploid genome, as there are two copies per genome, to give a final telomere length in kb per diploid genome (O'Callaghan et al. 2008).

Statistical analysis

Analysis of variance (ANOVA) was used to compare mean telomere lengths between sexes and age classes, where mean telomere length was the dependent variable and the factors were sex and age class. Bonferroni post-hoc analysis was used to identify differences in mean telomere lengths between the age classes. Finally, an ANOVA was used to compare telomere lengths between states of sexual maturity. Statistical analyses were performed using SPSS 15.0 software.

Results

In total, telomere lengths were measured in 86 individual Australian sea lions. Total telomere lengths ranged from 16.49 to 387.85 kb per diploid genome. In general, the pups showed the greatest range in telomere lengths of the three age classes examined (Fig. 1). Mean total telomere lengths exhibited a general decline with increasing age (Fig. 1).

When the data were pooled across all age classes for each sex, no significant difference in the mean total telomere lengths between sexes was identified (ANOVA: $F_{1, 85}$ = 1.198, P = 0.277). As there was no significant difference in mean total telomere length between the sexes, the data were pooled across sexes for each age class and were shown to be significantly different (ANOVA: $F_{2, 85}$ = 3.167, P = 0.047). Bonferroni post-hoc analysis determined that the adult age class had significantly smaller mean total telomere lengths than the pups (P = 0.021) and the juveniles (P = 0.028). However, there was no significant difference between the pup and juvenile age classes.

In order to avoid the difficulties associated with differentiating between large pups and small juveniles (McIntosh 2007), these animals were pooled as *immature* animals (n = 65) and compared with the *mature* adult females (n = 21). A significant difference between the two groups was found (ANOVA: $F_{1, 85}$ = 6.003, P = 0.016).

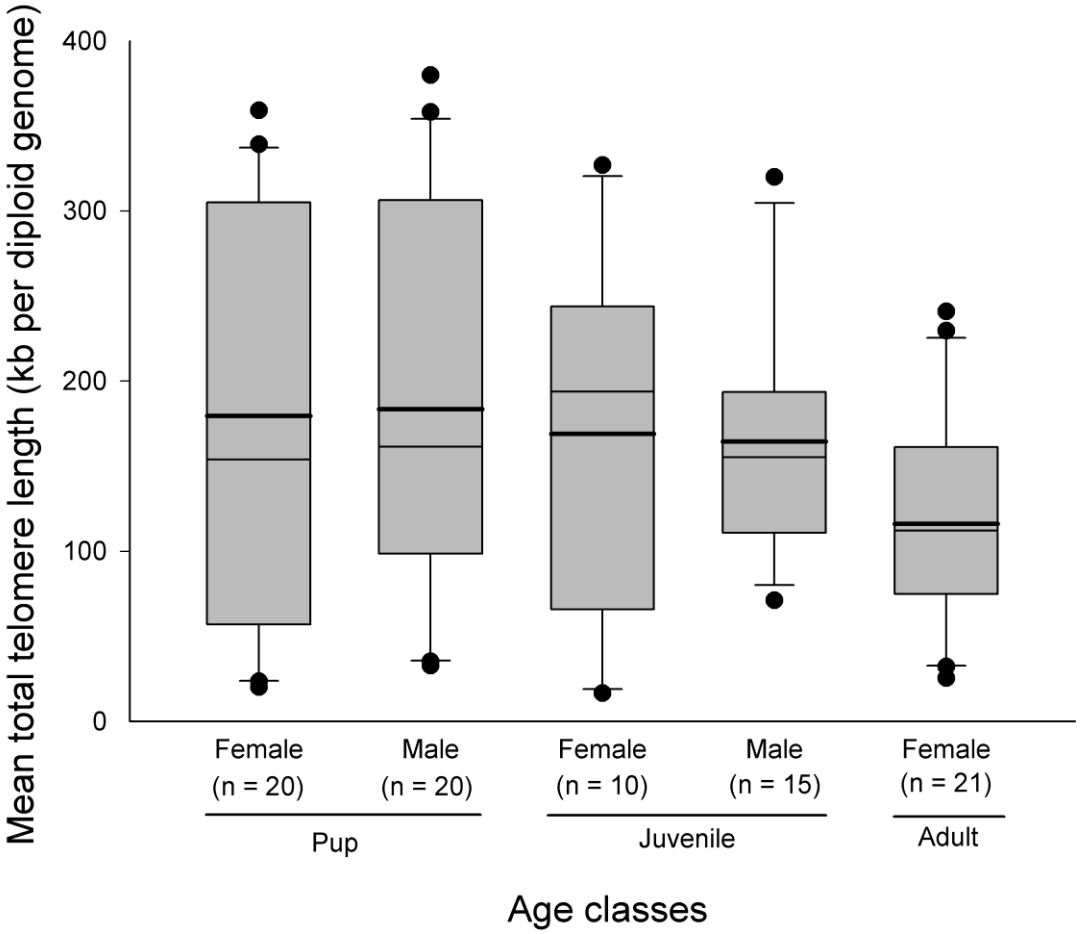

Figure 1. Comparisons of mean total telomere lengths between age classes, separated by sex, of the Australia sea lion, *Neophoca cinerea* (sample sizes in parenthesis). The boxes represent the ranges of total telomere lengths measured. The mean and median total telomere lengths are shown by the thick and thin horizontal lines in the box, respectively. The tails are ± the standard errors of the mean. (●) denote outlying values.

Discussion

We identified a general decline in telomere length with age in the Australian sea lion. Specifically, the mean total telomere length of adults was significantly shorter than for pups and juveniles. Furthermore, mean total telomere lengths in *mature* animals, which comprised the female adults, were significantly shorter than for *immature* animals, which comprised both pups and juvenile males and females. These findings fit the generalised mammalian pattern of telomere length attrition with age. Mean total telomere length measurements presented here are within the same size range as those reported in humans and had comparable variance in telomere lengths within age classes to other species of mammals (O'Callaghan et al. 2008; Thomas et al. 2008). This study marks the first such reported finding for a pinniped species.

The adult age class comprised sexually mature females that were identified as such because they were all observed suckling pups. The average age at which a female Australian sea lion reaches sexual maturity is 5 years (Ling et al. 2006; McIntosh 2007) and they may continue to breed until 26 years (McIntosh 2007). The age of the adults that we sampled ranged widely, with younger animals being small and short with well-preserved teeth and older animals being large and long with worn teeth (McIntosh 2007), indicating they represented the spectrum of ages. Despite this range in age among the adults, the variance of telomere lengths was smaller in adults compared with pups and juveniles.

The pup age class consisted of animals with less than one year difference in ages and had much larger variance in telomere length. The variance of telomere lengths reported in pups may be the result of: (i) rapid somatic growth of pups in the first year of life (Bell et al. 1997; McIntosh 2007); (ii) differences in the length of telomeres at birth (Monaghan & Haussmann 2006); and (iii) marked variance in the rate of change in telomere length between individuals (Bize et al. 2009). The observed variance is somewhat characteristic of the heterogeneous nature of telomeres between individuals in a typical mammalian population (Takubo et al. 2002; Monaghan & Haussmann 2006). A similar circumstance may exist in the juvenile age class, with the added complication of a greater range of ages included (1.5 to 5 years) compared with pups (0 to 1.5 years). These results indicate that there is a greater variance in the rate of decline in telomere lengths in younger age classes compared with older age classes. As animals

become older, the rate of decline in telomere length reduces and tends to plateau, thus compressing the overall telomere lengths in older age classes. When Australian sea lions were separated by their state of sexual maturity (i.e. 0 to 5 years and 5+ years), mature animals had significantly shorter mean total telomere lengths. Overall, significant telomere length reduction may occur upon or before the onset of sexual maturity. This is the general pattern of telomere length loss also seen in humans, with children to the age of 5 years undergoing the most rapid phase of telomere length change (Frenck et al. 1998; Rufer et al. 1999; Zeichner et al. 1999). The long-term measurement of telomere lengths of individuals within the population, i.e. longitudinal sampling, is needed to discern the causes and consequences of the variability of telomere lengths between individuals observed in Australian sea lions. This may be viable in the Australian sea lion given that individuals are routinely tagged (Higgins 1993; Gales et al. 1997) and display a measure of philopatry (Campbell et al. 2008), and repeated tissue sampling on an annual basis may be undertaken.

The sex of the animals sampled did not influence telomere lengths in the juvenile Australian sea lion. However, differences in telomere length between sexes have been reported previously in other mammalian species, with females generally possessing longer telomeres than males (Coviello-McLaughlin & Prowse 1997; Jeanclos et al. 2000; Cherif et al. 2003). A similar circumstance would be expected among Australian sea lions, given that telomere length is inversely proportional to somatic growth and that adult males are markedly larger than adult females (Ling 1992; McIntosh 2007). However, due to logistical limitations we were unable to obtain samples from adult males to compare their telomere lengths with adult females. Therefore, future studies may benefit from the inclusion of adult males.

It has been suggested that a measure of an individual‟s telomere length provides a potential indicator of the long-term survival and fitness of an individual (Hall et al. 2004; Bize et al. 2009). Longer telomeres are associated with extended individual longevities (Jemielity et al. 2007) and an extended reproductive lifespan (Keefe & Liu 2009). Natural selection theory predicts that young Australian sea lions with relatively short telomeres are „selected“ against and are removed from the population (Cawthon et al. 2003) and telomere length variation should gradually decrease over time in a

population or species. This potentially also explains why the adult females had less variability in the telomere length measurements.

These findings confirm that Australian sea lions share the same general pattern of declining telomere length with age as reported for other terrestrial mammals such as dogs (Nasir et al. 2001; Yazawa et al. 2001; McKevitt et al. 2002), cats (Brümmendorf et al. 2002; McKevitt et al. 2003) and humans (Hastie et al. 1990). We have shown that telomere lengths can be used to discriminate between broad age classes, specifically between pups and adults, juveniles and adults, or in a more collective sense between immature and mature animals. The application of telomere lengths as an age determinate requires considerable development to refine the scale of the age estimates derived, which will require the use of known-aged individuals. Nonetheless, telomeres have the potential to become valuable tools in molecular ecology, providing a non-invasive way of rapidly assessing the age structure of free-living populations. This tool may also be beneficial in assessing compliance in demographically based harvest management systems in exploited pinniped populations (DFO 2009), the importance of which cannot be understated, given the growing social and political pressure regarding the harvest of marine mammal populations.

“[Aging] an unsolved problem of biology”

…Sir Peter Medawar (1952)

Chapter Five

Rate of telomere length change with age in fishes correlate with species variation in longevity

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Text in manuscript

Statement of Authorship

In this chapter, Christopher Izzo collected the specimens, performed the TRF assays and animal ageing, analysed the data and wrote the manuscript. Bronwyn Gillanders and Stephen Donnellan assisted in the synthesis of the manuscript.

Certification that the statement of contribution is accurate.

Christopher Izzo (Candidate)

Signed *Date* 07/01/2010

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

Bronwyn M. Gillanders

Signed *Date* 07/01/2010

Stephen C. Donnellan

Signed *Date* 07/01/2010

Chapter Five Preface

Chapter Abstract

A relationship between the rate of telomere length change with age and species longevity has been observed in birds and mammals, with long-lived species experiencing less rapid telomere length sequence loss relative to short-lived species. We test whether fishes show a similar pattern. We calculated the rates of telomere length change with chronological age in 15 teleost and chondrichthyan species. These data were then collated with the available telomere length-at-age fish literature to assess correlations between species longevity telomere lengths. We found that the rate of telomere length change was inversely correlated to species longevity, with short-lived species experiencing rapid telomere length attrition relative to longer lived species. No correlations between longevity and the telomere lengths at birth were observed. These findings suggest that the rate of telomere length change is an important determinant of species longevity, as this rate is equivalent to the rate that the shortest telomere reaches a critical length signalling cellular senescence.

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Chapter Five

Telomere lengths of fishes correlate with species variation in longevity

Introduction

Globally there are an estimated 30,000 plus species of extant teleosts (bony fish) and chondrichthyans (sharks, rays, skates, and chimaeras) (Froese & Pauly 2008). As a taxonomic group, the fishes are unique in the breadth of diversity of their life history patterns and longevities (Bone et al. 1995). Longevities of fish can exceed 100 years, with the longest-lived species being the rockeye roughfish (*Sebastes aleutianus* Jordan & Evermann) reaching a staggering 205 years of age (Cailliet et al. 2001). This is in stark contrast to some genera of the killifishes (Order Cyprinodontiformes), which are represented by "annual" species, i.e. longevities < one year (Bone et al. 1995; Valdesalici & Cellerino 2003).

Identifying those mechanisms which contribute to the observed variation in longevity among species is fundamental to understanding inter-specific variability in rates of reproduction and mortality (Rose 1991; Mangel & Abrahams 2001), as well as providing an insight into the evolution of life history strategies (Stearns 1992; Haussmann & Mauck 2008). Increasing evidence indicates that telomeres play an important role in organismal aging and growth, as well as variation in longevity (von Zglinicki 2002; Monaghan & Haussmann 2006; Haussmann & Mauck 2008).

Telomeres are tandem non-coding repeated nucleotide sequences that cap the ends of all eukaryotic chromosomes (Blackburn 1990). In normal somatic cells, telomeres undergo processes that shorten their lengths (Blackburn 1990). Each time a cell divides, the telomeric DNA on its chromosomes gets shorter, until a crucial length is reached, whereby the telomere becomes dysfunctional (Allsopp & Harley 1995; Hemann et al. 2001). Telomere dysfunction results in the cessation of normal cellular division, which leads to apoptosis (cell death), or replicative senescence, whereby the cell continues to function but is incapable of dividing (Campisi et al. 2001; Hemann et al. 2001; Nosek et al. 2004).

While no correlation between absolute telomere lengths and longevity in a range of vertebrate species has been identified (Pardue & DeBaryshe 1999; Goyns & Lavery 2000; de Magalhães & Toussaint 2004), the rate at which telomere lengths change over a species lifespan has been shown to be an important correlate of species longevity (Monaghan & Haussmann 2006). In birds and mammals, the rate of telomere length change with chronological age is correlated inversely with longevity (Haussmann et al. 2003; Vleck et al. 2003). Here, we test whether fish (teleosts and chondrichthyans) show a similar pattern.

In addition, the mean telomere length at birth, referred to here as the initial telomere length, may also be important in assessing correlative links between telomeres and aging (Vleck et al. 2003). Therefore, in order to assess the role of telomeres as an underlying contributor to the observed variation in fish longevities, we calculated this measure of telomere length in a range of fish species.

Methods

Muscle biopsy samples were obtained from specimens from 15 species of fishes (Table 1). We used muscle as this tissue shows a relationship between telomere length and age in teleosts (Tsui 2005; Hatakeyama et al. 2008; Hartmann et al. 2009). Estimates of telomere length were made using the Telo TAGGG Telomere Length Assay (Roche Diagnosis). Briefly, restriction enzyme digested DNA fragments were separated by gel electrophoresis and transferred to a charged nylon membrane by Southern blotting. The membrane was hybridised to a digoxigenin-labelled probe complementary to the telomeric repeat, then incubated with a digoxigenin-specific antibody bound to an alkaline phosphatase. The hybridised DNA was visualised through incubation in a chemiluminescent CDP-Star substrate and exposure to X-ray film. Average telomere lengths were calculated by comparing the relative telomeric signal to a molecular weight standard.

Table 1. Summary of chondrichthyan and teleost species investigated for relations between the telomere length and age.

Class	Order	Family	Species	Common name	Annual increment validation
Chondrichthyes					
Subclass Elasmobranchii	Heterodontiformes	Heterodontidae	*Heterodontus portusjacksoni*	Port Jackson shark	1
	Squaliformes	Squalidae	*Squalus megalops*	Piked spur-dog	2
	Myliobatiformes	Urolophidae	*Urolophus paucimaculatus*	Sparsely spotted stingaree	3
	Rajiformes	Rajidae	*Dentiraja lemprieri*	Thornback skate	annuli assumed
	Rhinobatiformes	Rhinobatidae	*Trygonorrhina dumerilii*	Southern fiddler ray	4
Subclass Holocephali	Chimaeriformes	Callorhinchidae	*Callorhinchus milii*	Elephant shark	annuli assumed
Osteichthyes					
Class Sarcopterygii	Ceratodontiformes	Ceratodontidae	*Neoceratodus forsteri*	Australian lungfish	5
Class Actinopterygii	Cypriniformes	Cyprinidae	*Cyprinus carpio*	Common carp	6
	Perciformes	Carangidae	*Pseudocaranx wrighti*	Silver trevally	annuli assumed
			Trachurus novaezelandiae	Southern yellowtail scad	7
		Mullidae	*Upeneichthys vlamingii*	Southern goatfish	annuli assumed
		Percichthyidae	*Macquaria ambigua*	Golden perch	8
		Sparidae	*Chrysophrys auratus*	Snapper	9
	Scorpaeniformes	Platycephalidae	*Platycephalus bassensis*	Sand flathead	annuli assumed
	Tetraodontiformes	Monacanthidae	*Thamnaconus degeni*	Degen‟s leatherjacket	annuli assumed

Data sourced from: [1]Tovar-Ávila et al. (2008); [2]Braccini et al. (2007); [3]White et al. (2005); [4]Izzo & Gillanders (2008); [5]Known age specimens; [6]Vilizzi & Walker (1999); [7]Horn (1993); [8]Anderson et al. (1992); [9]Francis et al. (1992).

Telomere length measurements were calibrated to age estimates based on counts of growth increments in the ageing structures (i.e. vertebrae, fin spines and otoliths). Individual structures were embedded in resin and sectioned with a lapidary saw. Sections were then mounted on a microscope slide and examined under a dissecting microscope. Multiple counts of increments were made from one calcified structure per specimen. Annual increment formation had been validated previously in eight of our study species (Table 1). For the remaining species, we made the explicit assumption that periodicity of increment formation was annual (Table 1).

We compiled previously reported teleostean telomere length-at-age data for: the black porgy (*Acanthopagrus schlegeli* Bleeker), the European sea bass (*Dicentrarchus labraxi* Linnaeus), the mangrove snapper (*Lutjanus argentimaculatus* Forsskal), the medaka (*Oryzias latipes* Temminck & Schlegel), and the bluefin killifish (*Nothobranchius rachovii* Ahl), as well as two laboratory strains of the turquoise killifish (*N. furzeri* Jubb) (Table 2). All fishes (i.e. 15 from our research plus 7 from the published literature) were then used to assess correlations between species longevity and telomere lengths.

Estimates of species longevity were obtained from FishBase (Froese & Pauly 2008), AnAge (de Magalhães et al. 2005) and selected references (Tables 2 & 3). As species longevity estimates are based on observed and, or extrapolated growth data and may potentially over- or under-estimate true species longevity, we also tested correlations between the rate of telomere length change and maximum observed total body length of the species (data obtained from FishBase).

Regression analysis was used to examine telomere length-at-age relations for each species. The slopes of the regressions provide a mean rate of telomere length change throughout the species‟ lifespan. Analysis of covariance (ANCOVA) was used to compare rates of telomere length change between species, where mean telomere length was the dependent variable, chronological age was the covariate and species was a fixed factor. The *Y*-intercepts of the regression analyses provided a mean initial telomere length for each species. Non-linear logarithmic regression analysis (NLLRA) was used to assess correlations between the species longevity and initial telomere lengths.

Results

Telomere lengths decreased significantly with chronological age in four species of teleosts: the flathead (*Platycephalus bassensis* Cuvier), the snapper (*Chrysophrys auratus* Forster), Degen‟s leatherjacket (*Thamnaconus degeni* Regan), and the southern goatfish (*Upeneichthys vlamingii* Cuvier) (Fig. 1A – D, Table 3). We identified significant telomeric elongation with age in the carp (*Cyprinus carpio* Linnaeus) (Fig. 1E). The rates of telomere length change, i.e. the slopes of the regressions, between these five species were significantly different (ANCOVA: $F_{5, 49} = 71.990$, $P < 0.0001$). When *C. carpio* was excluded from the analysis, rates of telomere length change remained significantly different between the four teleost species (ANCOVA: $F_{4, 36} = 49.926$, $P < 0.0001$).

Telomere lengths did not change significantly with chronological age within the remaining four teleost species examined: the southern yellowtail scad (*Trachurus novaezelandiae* Richardson), the silver trevally (*Pseudocaranx wrighti* Whitley), and the golden perch (*Macquaria ambigua* Richardson), nor in the lungfish (*Neoceratodus forsteri* Krefft); as well as in all six species of chondrichthyans (Table 3).

The annual rate of telomere length change was related to species longevity (NLLRA: $r^2 = 0.54$, $P < 0.0001$), and total body length (NLLRA: $r^2 = 0.46$, $P < 0.0001$) (Fig. 2). Those short-lived species, which attain smaller total body lengths, experienced more rapid telomere length loss than the long-lived, larger species. Initial telomere lengths were not correlated to species longevity (NLLRA: $P = 0.148$), or total body length (NLLRA: $P = 0.330$) (Fig. 3A & B).

Table 2. Comparisons of teleost telomere length-at-age regression parameters. *n* is the sample size, ML is the maximum species longevity; TL is the maximum total body length (mm); TROC is the annual telomere length rate of change (in bp per year); and ITL is initial telomere length (in kb). n/a: where no data available.

Species	*n*	ML (yrs)	Age range	TL (mm)	r^2	*P*-value	TROC	ITL
Acanthopagrus schlegeli[1]	18	7[1]	1 – 3	500*	0.21	0.059	–441.4	5.56
Dicentrarchus labrax[2]	24	15**	1 – 7	1030*	0.03	> 0.05	32	3.44
Lutjanus argentimaculatus[1]	27	18*	0.2 – 3	1500*	0.15	< 0.05	–215	5.26
Nothobranchius furzeri - MZM-0403 strain[3]	16	0.5[3]	0.09 – 0.4	65*	n/a	0.004	–3900[α]	6.08[α]
Nothobranchius furzeri - GRZ strain[3]	38	0.3[3]	0.04 – 0.3	65*	n/a	> 0.05	–743[α]	6.61[α]
Nothobranchius rachovii[4]	15	0.7[4]	0.08 – 0.6	60*	n/a	< 0.001	–4260[α]	7.77[α]
Oryzias latipes[5]	67	5**	1 – 4	32*	0.33	0.010	–1440	15.6

Data sourced from: *FishBase; **AnAge; [1]Tsui (2005); [2]Horn et al. (2008); [3]Hartmann et al. (2009); [4]Hsu et al. (2008) ; [4]Hatakeyama et al. (2008). [α]Calculated by performing a linear regression on the reported data.

Table 3. Comparisons of teleost and chondrichthyan telomere length-at-age regression parameters. *n* is the sample size, ML is the maximum species longevity; TL is the maximum total body length (mm); TROC is the annual telomere length rate of change (in bp per year); ITL is initial telomere length (in kb); and 0^+ indicates young of the year animals.

Species	*n*	ML (yrs)	Age range	TL (mm)	r^2	*P*-value	TROC	ITL
Chondrichthyes								
Callorhinchus milii	10	6**	0^+ – 5	1250*	0.01	0.766	106	24.59
Heterodontus portusjacksoni	20	35[1]	1 – 16	1650[2]	0.37	0.194	62	23.21
Squalus megalops	10	75**	0^+ – 6	710*	0.43	0.079	368	24.12
Dentiraja lemprieri	10	12[4]	0^+ – 10	600[2]	0.006	0.836	29	21.42
Trygonorrhina dumerilii	16	15[3]	0^+ – 9	1260[2]	0.11	0.204	–197	26.67
Urolophus paucimaculatus	9	12**	0^+ – 4	570*	0.02	0.736	88	24.39
Osteichthyes								
Chrysophrys auratus	11	35**	2 – 10	1300*	0.79	< 0.0001	–797	12.61
Cyprinus carpio	13	38*	2 – 8	1200*	0.79	< 0.0001	1177	4.62
Macquaria ambigua	24	20**	4 – 11	760*	0.02	0.535	–30	6.14
Neoceratodus forsteri	6	65*	0^+ – 20	1700*	0.03	0.452	175	24.22
Platycephalus bassensis	10	23[5]	1 – 6	460*	0.78	0.002	–746	12.19
Pseudocaranx wrighti	11	13[7]	1 – 8	225[8]	0.01	0.795	–40	4.06
Thamnaconus degeni	10	18[6]	8 – 18	280*	0.76	0.001	–623	15.31
Trachurus novaezelandiae	10	25*	0^+ – 5	500*	0.03	0.639	78	4.72
Upeneichthys vlamingii	9	10[4]	1 – 6	350*	0.85	0.003	–400	5.48

Data sourced from: *FishBase; **AnAge; [1]Izzo (2005); [2]Last & Stevens (2009); [3]Izzo & Gillanders (2008); [4]eldest specimen examined during this study; [5]Koopman & Morison (2002); [6]Svane et al. (2007); [7]Ratnasingham & Hebert (2007); [8]Platell & Potter (2001).

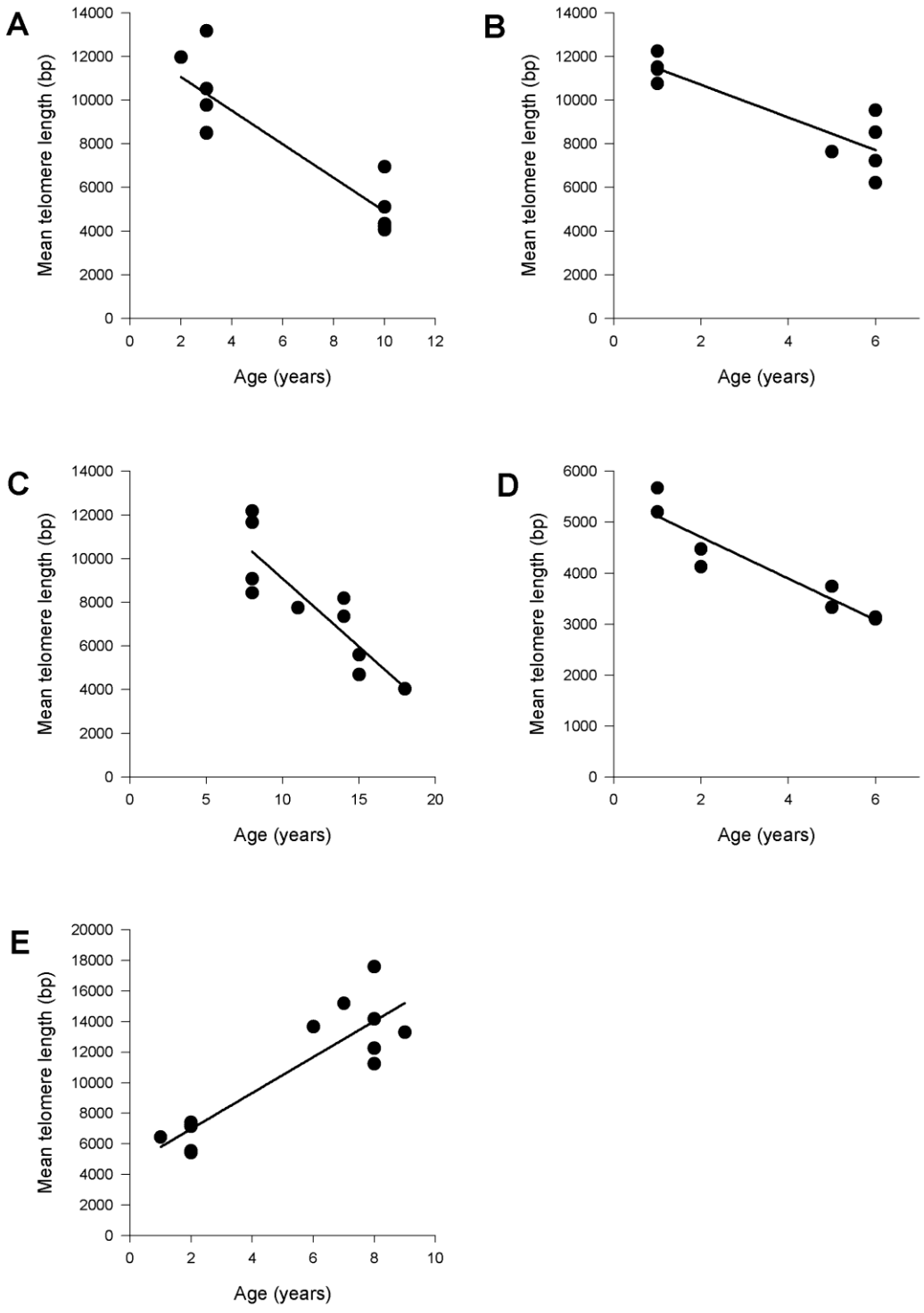

Figure 1. Telomere length-at-age relations in five species of teleost fishes. (A) *Chrysophrys auratus*; (B) *Platycephalus bassensis*; (C) *Thamnaconus degeni*; (D) *Upeneichthys vlamingii*; and (E) *Cyprinus carpio.* The solid lines are the regression lines of best fit. Note that the *X*- and *Y*-axes vary by species.

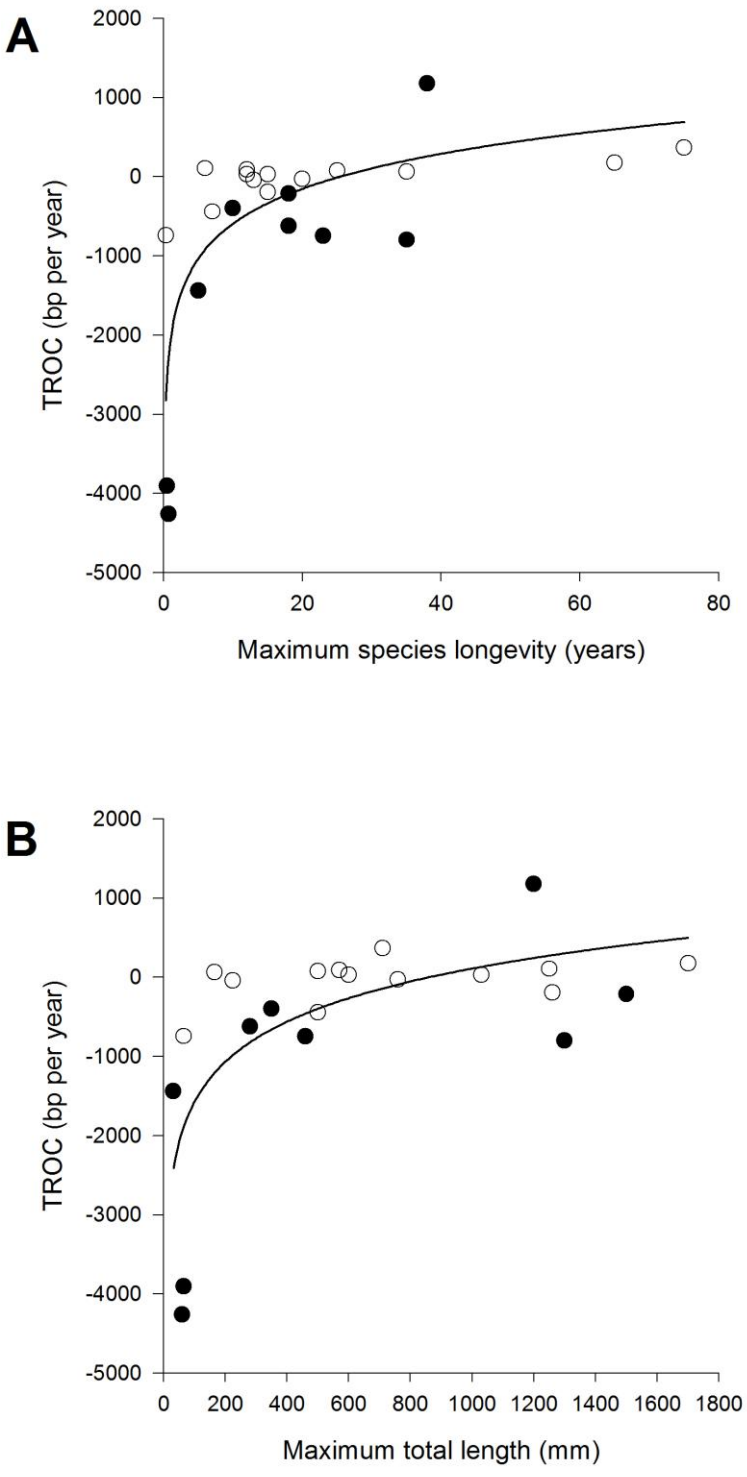

Figure 2. Relations between the rate of change in telomere lengths (TROC) (base pairs lost/gained annually) and (A) maximum species longevity; (B) maximum total body length. Where: ● species with significant telomere length-at-age relations; and ○ for all species combined. The solid line is the non-linear logarithmic regression lines of best fit for all species combined.

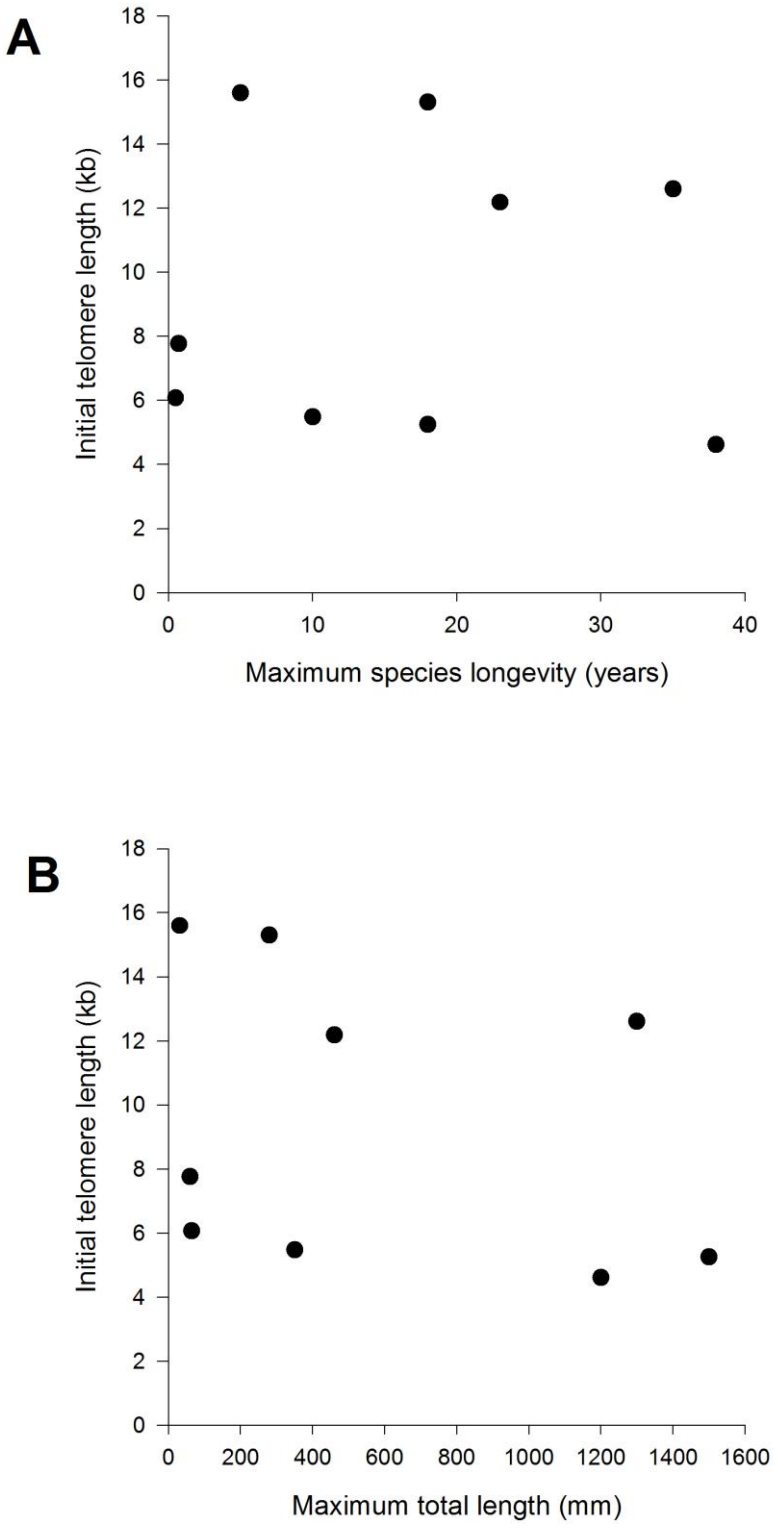

Figure 3. Relations between initial telomere lengths and (A) maximum species longevity; and (B) maximum total body length.

Discussion

We have demonstrated that the rate of telomere length change is strongly correlated with species longevity. In contrast, initial telomere lengths were not correlated with species longevity in fish. Fish species that undergo telomere length loss at a slower rate (and in the unusual case of the *C. carpio*, elongate telomeres annually) are longer lived than those species that experience relatively rapid rates of telomere length attrition. This is best illustrated in the annual teleost species, the killifishes (genera *Nothobranchius*), which despite having longevities of approximately 6 months experience the most dramatic telomere length attrition – in excess of 3 kb per year (extrapolated from Hsu et al. 2008; Hartmann et al. 2009). These findings are consistent with the observed patterns of telomere length change in a range of avian and mammalian species (Haussmann et al. 2003; Vleck et al. 2003).

The rate of telomere length change is equivalent to the rate that telomeres reach a critical length signalling cellular senescence (Hemann et al. 2001; Haussmann et al. 2003; Vleck et al. 2003). Therefore, a better understanding of the molecular mechanisms that may contribute to variable patterns of telomere length change in fish, i.e. factors that act to restore and shorten telomeres, may assist in elucidating the evolution of variation in species longevities (Monaghan & Haussmann 2006).

Long-lived species may possess mechanisms, such as persistent expression of telomerase, that maintain telomere lengths throughout their life time. Telomerase is a ribonucleoprotein enzyme, which functions as a reverse transcriptase, using an internal RNA template to maintain and, or extend the telomeric sequence (Bryan & Cech 1999). In fish, telomerase is constitutionally expressed at high levels in somatic tissues (Barker et al. 2000; Yoda et al. 2002; Elmore et al. 2008; Lund et al. 2009), suggesting the potential to maintain or elongate telomere lengths (Bodnar et al. 1998). Yet, despite considerable telomerase activity in multiple tissues examined in two short-lived teleost species, *N. furzeri* and *O. latipes*, telomere length attrition has been observed (Elmore et al. 2008; Hatakeyama et al. 2008; Hartmann et al. 2009). This suggests that the expression of telomerase does not necessarily ensure telomere length maintenance in fish. Indeed, the reverse transcriptase component of telomerase has other roles (Saretzki 2009), e.g. embryonic anterior–posterior axis formation (Park et al. 2009), and telomere lengthening activity could be controlled by expression of the separately genetically encoded RNA template component of telomerase or the large number of proteins that comprise the teleosome (Neumann & Reddel 2002).

On the other hand fish may show inter-specific variation in resilience to deleterious factors which damage DNA (Vleck et al. 2003), such as oxidative damage (von Zglinicki et al. 2000), or variable rates of cell division (Allsopp et al. 1995), both of which have been shown to cause significant telomere shortening, expediting cellular senescence. A better understanding of how these intrinsic and extrinsic factors affect rates of telomere length change within and between species will assist in understanding what part telomeres play in determining fish longevities.

These findings indicate that the rate of telomere length change in fishes is highly correlated to species longevity. We have shown that short-lived fish species undergo greater telomere length attrition than their longer-lived counterparts. The rate of change corresponds to the rate at which the shortest telomere reaches a critical length initiating cellular senescence (Hemann et al. 2001). A greater understanding of the mechanisms that contribute to intra-specific variability of rates of telomere length change in fishes may elucidate the factors that moderate species longevities.

“Nothing in biology makes sense except in the light of evolution”

…Theodosius Dobzhansky (1973)

Chapter Six

Recent evolution of telomere length change with age in gnathostomes

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Text in manuscript

Statement of Authorship

In this chapter, Christopher Izzo collected the specimens, performed the telomere length measurements and increment ageing. In addition, C. Izzo completed the phylogenetic analysis and the ancestral state reconstruction analysis, synthesised the data and wrote the manuscript. Stephen Donnellan aided in the phylogenetic and reconstruction analysis and manuscript synthesis. Bronwyn Gillanders contributed to the manuscript preparation.

Certification that the statement of contribution is accurate.

Christopher Izzo (Candidate)

Signed *Date* 07/01/2010

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis.

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Signed *Date* 07/01/2010

Bronwyn M. Gillanders

Signed *Date* 07/01/2010

Chapter Six Preface

Chapter Abstract

Telomeres naturally undergo changes in length with chronological age in the gnathostomes (jawed vertebrates). Information outlining the phylogenetic distribution of patterns of telomere length change with age is limited to a few “advanced” teleosts and tetrapods, impeding determinations of the evolutionary origin(s) of this trait. We test alternative hypotheses for the evolution of telomere length change with age by ancestral state reconstruction in a set of 40 gnathostomes for which we expanded the sampling of chondrichthyans and teleosts. We found that telomere length did not change with age in the common ancestor and has since evolved independently. Birds and teleosts showed the highest rates of evolutionary lability of patterns of telomere length change with age through repeated transitions from and reversions to the ancestral state. Correlative analyses involving broad taxonomic comparisons are unlikely to identify the ecological or physiological factors responsible for these transitions, but will likely be studied best within lineages with a recent history of transitions.

Chapter Acknowledgements

We thank SARDI Aquatic Sciences and Jean Joss for assistance in specimen collection, and Adam Skinner for assistance with the ancestral state reconstruction. Animal procedures were approved by the University of Adelaide animal ethics committee (Project No. S-022-2006). This research was supported by funding from Sea World Research and Rescue Foundation Inc., the Australian Academy of Science, and The Sir Mark Mitchell Research Foundation.

Chapter Six

Recent evolution of telomere length change with age in gnathostomes

Introduction

Telomeres are short repeated nucleotide sequences that cap the ends of all eukaryotic chromosomes. Telomeres naturally undergo processes that either change or maintain their lengths (Blackburn 1990). Telomere length has been suggested as a biomarker of cellular aging (Harley et al. 1992), which led to the paradigm, based on *in vivo* and *in vitro* mammalian research, that telomere length declines with organismal age (Hastie et al. 1990) and could be used as a novel determinate of chronological animal age (Haussmann & Vleck 2002).

More recently, relationships between telomere length and age have been assessed in a wide range of vertebrate species including an amphibian, avian and non-avian reptiles, mammals and teleost fishes (Chapter Five; see Table A1, Appendix One); these studies show that the generalised view of telomere length decline with age is not shared universally among jawed vertebrates (gnathostomes). Three different patterns of telomere length change with age have been identified: (i) a common pattern of an overall decline in telomere length with age (Chapter Four); (ii) an uncommon pattern of an increase of telomere length with age (Chapter Three); and finally, (iii) the absence of a significant change of telomere length with age (Chapter Two).

By expanding the taxonomic focus of telomeric research, paradigms in telomere biology based on limited taxonomic sampling require rethinking to be inclusive of all taxa. Furthermore, the variety in patterns of telomere length change with age provides an opportunity to identify the ecological or physiological factors involved in selecting for these transitions.

However, before we seek to identify the selection factors responsible for these transitions between states of telomere length change with age, we first need to identify the overall evolutionary patterns of telomere length change, in particular, to allow targeting of groups with a recent history of state transition. Here we test two hypotheses for the evolution of

telomere length change with age in gnathostomes: (i) telomere length change with age evolved early and has since disappeared in several instances throughout the history of the gnathostomes; or alternately (ii) this phenomenon was not present ancestrally and has evolved independently in different gnathostome lineages with some secondary losses.

To test which of these hypotheses best fits the phylogenetic distribution of patterns of telomere length change, we assessed the relationships between telomere length change and age in the chondrichthyes (sharks, rays, skates, and chimaeras), sampled further taxa from a more diverse range of teleost lineages, and the lungfish the sister lineage to the tetrapod vertebrates. We then reconstructed the ancestral state of telomere length change with age using maximum parsimony (MP) and maximum likelihood (ML) methods.

Methods

Sampling

Samples of muscle, which have been shown to be a suitable tissue for telomere ageing in species of teleosts (Tsui 2005; Hatakeyama et al. 2008), were obtained from 15 fish species across 11 orders.

Molecular methods

DNA was isolated following prescribed methods (Gentra Systems). Telomere length from ~1 $\mu g\ \mu l^{-1}$ of genomic DNA was estimated using the Telo TAGGG Telomere Length Assay (Roche Diagnosis). DNA was co-digested with restriction enzymes *HinfI* and *RsaI* for 2 h at 37 °C. The digested DNA fragments were separated by constant field gel electrophoresis and transferred to a positively charged nylon membrane by Southern blotting. The membrane was hybridised to a digoxigenin labelled telomeric probe for 3 h at 42 °C and incubated with a digoxigenin-specific antibody bound to alkaline phosphatase.

The telomeric probe was visualised through incubation in chemiluminescent CDP-Star substrate. The membrane was then exposed to X-ray film, which, once developed, was scanned with the analytical software Quantity One (Bio-Rad), where average telomere lengths were calculated.

Ageing fishes

In order to calibrate telomere length measurements to animal age, counts of growth increments in the ageing structures (vertebrae, fin spines and otoliths) of the specimens were

made. Calcified structures were cleaned of excess tissues then embedded in an epoxy resin and cut into approximately 300 μm sections with a lapidary saw. Sections were then mounted onto a microscope slide and examined under a dissecting microscope with a transmitted light source.

Multiple counts of increments were made from one calcified structure per specimen. Increments were defined as paired opaque and translucent circuli (Cailliet et al. 2006). Counts were made without prior knowledge of the size, sex or previous count of the specimen. If the two counts varied, a third count was made. If the third matched either of the previous two it was taken as the consensus increment number. If no consensus was reached, then that specimen was excluded.

Age estimates presented here are based on counts of growth increments, with the exception of the *Neoceratodus forsteri* (Krefft) samples, which were of known age. Annual increment formation had been validated previously in eight of our study species (Table 1). For the remaining six species, we made the explicit assumption that periodicity of increment formation was annual, i.e. one increment = one year. For each species we tested the correlation between telomere length change and age with regression analysis.

Ancestral state reconstruction

Relationships between telomere length and age among representative gnathostome species were collated from published studies (see Table A1, Appendix One). Each taxon was categorised as displaying one of three states of telomere length change with age: (0) no significant relationship; (1) decreasing telomere length; and (2) increasing telomere length. We selected taxa for reconstruction of telomere length with age transitions to represent all major gnathostome lineages with telomere length with age data and we also included taxa showing variation in states within any major lineage (Table 1). The 40 taxa used in our data set are detailed in Table A1 (Appendix One).

To construct a phylogenetic tree for ancestral state reconstruction we combined nucleotide sequences from five mitochondrial genes (*CO1*, *cytochrome b*, *ND4*, *12S rRNA* and *16S rRNA*) from GenBank and our own data for representatives of each of the 40 taxa in our data set (see Table A1, Appendix One). Where nucleotide sequence data from the taxon with telomere length with age data were not available we included sequence data from the next most closely related taxon within that genus, family or order according to availability.

Nucleotide sequences were aligned with Clustal X2 (Larkin et al. 2007) and improved by eye where required, resulting in 5990 bp of aligned sequence (the alignment is available on request from the authors). Sources of data used are presented in Table A2 (Appendix One) in the Supporting Information. We used the maximum likelihood (ML) program RAxML version 7.0.3 (Stamatakis 2006) to find the best ML tree based on a constraint tree of generally accepted gnathostome phylogenetic relationships (Janvier 1997; Nunn & Stanley 1998; Venkatesh et al. 2001; Hackett et al. 2008) (see Fig. A1, Appendix One).

The constraint tree node specifications were as in the best tree found (see Fig. A1, Appendix One), except for the Rajiformes where a trichotomy joined the three representatives (Nelson 2006). The Perciformes, Scorpaeniformes and Tetraodontiformes where two polytomies were used to represent uncertainties in relationships among the three orders, including paraphyly of Perciformes and Scorpaeniformes (Nelson 2006) and uncertainty in relationships among the perciform families (Nelson 2006) and a trichotomy among (Passeriformes, Charadriformes), (Pelecaniformes, (Sphenisciformes, Procellariformes)) and Apodiformes according to Hackett et al. (2008).

Ancestral state reconstruction by maximum parsimony (MP) and likelihood (ML) methods was implemented in Mesquite version 2.01 (Maddison & Maddison 2009) fitted on the best tree as generated by RAxML. Maximum parsimony reconstruction sought to identify the ancestral state whilst minimising the number of transitions between telomere length change states.

The MP analysis was conducted under the *unordered* states assumption; that is, a transition between any two states of telomere length change is counted a single change, e.g. a transition from state 0 to 2 was counted as one change (Maddison & Maddison 2009).

Table 1. Species with prior annual increment validation.

Common name (*Species*)	**Reference**
Golden perch (*Macquaria ambigua*)	Anderson et al. (1992)
Snapper (*Chrysophrys auratus*)	Francis et al. (1992)
Yellowtail scad (*Trachurus novaezelandiae*)	Horn (1993)
Common carp (*Cyprinus carpio*)	Vilizzi & Walker (1999)
Sparsely-spotted stingaree (*Urolophus paucimaculatus*)	White et al. (2005)
Piked spur-dog (*Squalus megalops*)	Braccini et al. (2007)
Southern fiddler ray (*Trygonorrhina dumerilii*)	Izzo & Gillanders (2008)
Port Jackson shark (*Heterodontus portusjacksoni*)	Tovar-Ávila et al. (2008)

The ML reconstruction method was then applied to identify the ancestral state that had the highest likelihood of evolving under a stochastic model of evolution. We used the Markov k-state one parameter model (*Mk1*) under the assumption that any particular change between the different states (i.e. from state 0 to 1 or state 2 to 1) is equally probable (Maddison & Maddison 2009), as the AsymmMk (Asymmetrical Markov k-state 2 parameter) model, which allows two rates of change was not implementable with a three state character. Conservatively, we accepted the more ambiguous determination for ancestral state reconstruction for a node when the MP and ML reconstructions differed.

Results

Telomere length decreased significantly with age in four of the nine species of teleosts examined, and significantly increased in length in *Cyprinus carpio* (Linnaeus) (Fig. 1, Table 1). In the remaining four teleosts and in all chondrichthyans, telomere length did not significantly change with age (Fig. 1, Table 1).

Ancestral character state reconstruction with MP indicated no relationship between telomere length and age was the most probable ancestral state for gnathostomes (Fig. 2: node 2). Of the four higher level nodes in the phylogeny, one has no relationship between telomere length and age as the reconstructed state i.e. 3 – the Chondrichthyes, and for the other three, 8 – the Osteichthyes, 9 – the Actinopterygii and 20 – the Sarcopterygii, the reconstructed state was ambiguous (Fig. 2). For the Osteichthyes and the Sarcopterygii the ML reconstruction was ambiguous (i.e. either state 0: no significant relationship, or state 1: decreasing telomere length) but MP inferred state 0 for both nodes. For the Actinopterygii, both methods inferred all three states as equally probable. Further analysis by ML reconstruction concurred with this finding. MP and ML values at all nodes are detailed in Table A3 (Appendix One).

All of the Chondrichthyes retained the ancestral gnathostome state. By contrast, a number of state transitions were inferred among the Actinopterygii. We identified between one and four reversals from state 1 to 0 in the Actinopterygii clade, the uncertainty being due to ambiguous reconstructions for nodes 13, 14, 15, 16, 17 and 18 by either or both ML and MP.

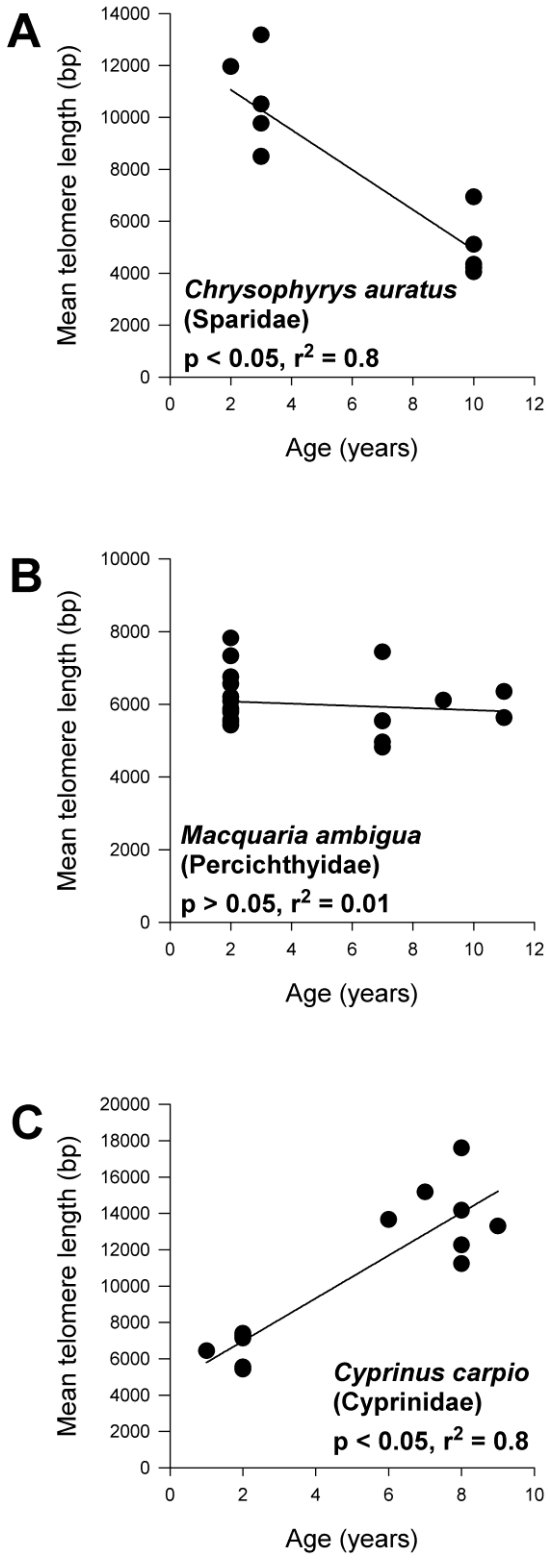

Figure 1. Three patterns of telomere length change in teleosts: (A) telomere length declines with age; (B) no significant change in telomere length with age; and (C) telomere length increases with age. Solid lines are the lines of best fit. Note that the *X*- and *Y*-axes vary by species.

Table 2. Comparisons of the regression analysis between the telomere length and age across 15 teleost and chondrichthyan species. State: (×) no significant change in telomere length with age; (–) telomere length declines with age; and (+) telomere length increases with age.

Species	***n***	r^2	***F***	***P***	**State**
Osteichthyes					
Chrysophrys auratus	11	0.794	34.760	< 0.0001	–
Cyprinus carpio	13	0.796	43.058	< 0.0001	+
Macquaria ambigua	24	0.018	0.396	0.535	×
Neoceratodus forsteri	6	0.374	2.431	0.194	×
Platycephalus bassensis	10	0.779	24.649	0.002	–
Pseudocaranx wrighti	11	0.008	0.071	0.795	×
Thamnaconus degeni	10	0.758	25.018	0.001	–
Trachurus novaezelandiae	10	0.033	0.241	0.639	×
Upeneichthys vlamingii	9	0.845	27.270	0.003	–
Chondrichthyes					
Callorhinchus milii	10	0.012	0.095	0.766	×
Dentiraja lemprieri	10	0.006	0.046	0.836	×
Heterodontus portusjacksoni	20	0.032	0.592	0.452	×
Squalus megalops	10	0.426	4.448	0.079	×
Trygonorrhina dumerilii	16	0.113	1.775	0.204	×
Urolophus paucimaculatus	9	0.020	0.125	0.736	×

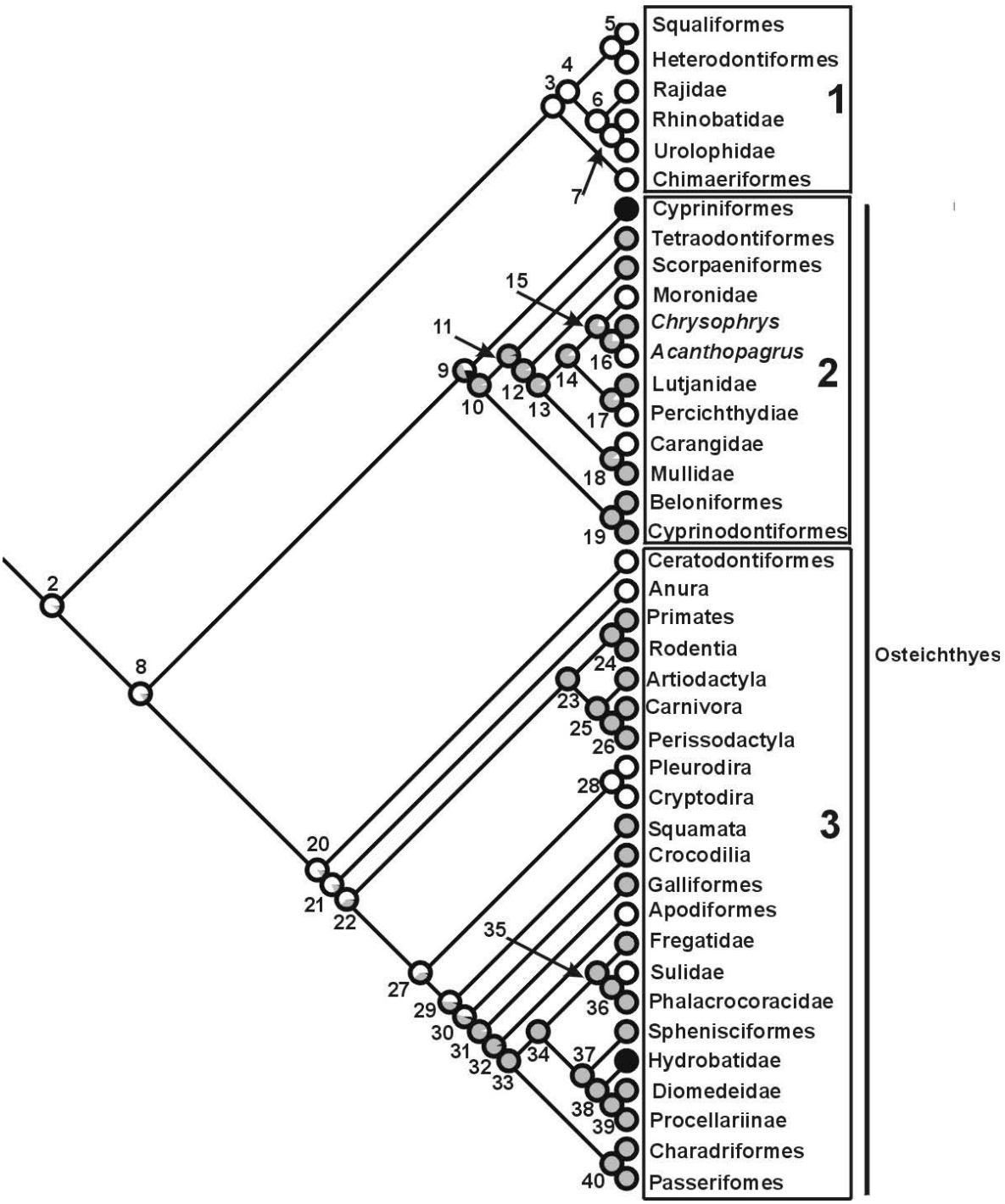

Figure 2. The distribution of the telomere length with age relationships within gnathostomes, where: ○ telomere length declines with age; ● telomere length increases with age; and ○ no significant change in telomere length with age (for sources of data see Table A1, Appendix One). The pie diagrams at the numbered nodes show the relative contribution to the ML estimate of each ancestral state of telomere length change with age (for relative contribution to ML and MP for each state at all nodes see Table A3, Appendix One). Numbered boxes indicate higher level taxa: (1) Chondrichthyes; (2) Actinopterygii; and (3) Sarcopterygii.

Furthermore, a transition from either state 0 or 1 to state 2 occurred either along the Cypriniformes lineage or ancestrally to the Actinopterygii clade. Basally within the Sarcopterygii, our analyses inferred ambiguous reconstructions (state 0 or 1) for the nodes 20, 21, 22, 27, 29 and 30. However within the bird clade, our analyses identified two reversals from state 1 to 0 and an unambiguous transition from state 1 to 2 within the Procellariiformes (Hydrobatidae).

Discussion

By documenting patterns of telomere length change with age for the first time in chondrichthyes and also doubling the representation of the range of teleost lineages sampled, we have elucidated the phylogenetic distribution of patterns of telomere length change with age among the major gnathostome lineages. Our findings reject the hypothesis that telomere length change with age evolved early in gnathostomes and has since reverted in some recent lineages. Our ancestral state reconstructions by two methods (MP and ML) shows that telomere length change with age could have evolved in the common ancestor of the Osteichthyes, or alternatively more recently in each of the common ancestors of the Actinopterygii, the mammals and the squamate/archosaur clade. We identified between three and six reversions to the ancestral state and two independent transitions to increasing telomere length with age in the Actinopterygii and the bird clades.

The biological correlates of the transition between no relationship with age and relationships with age have not been addressed previously. To date previous studies, primarily restricted to humans, rodents and birds may have confounded the causes and consequences of telomere length change (Aviv et al. 2003; Nakagawa et al. 2004).

A better way to determine the causative factors of telomere length change with age would be to study groups in which the transition from the ancestral state occurs repeatedly, which we have identified as the Actinopterygii and bird clades. This will allow a shift from broad based macro-evolutionary approaches to identify the causative factors in the evolution of telomere length with age to an analysis within recently evolved groups where the transition process is still occurring. However, life history traits that are fixed within a lineage, such as the high metabolic rate shared by all birds, do not provide a universal explanation of the polymorphism of patterns of telomere length change with age, as this trait is generally not shared among all species of fish (Glazier 2005). Similarly, life history traits that vary within a lineage, i.e. shifts from indeterminate to determinate growth patterns across species of fish

(King & McFarlane 2003) do not provide a broad explanation for shifts between patterns of telomere length with age, as all bird (and mammalian) species have a fixed determinate growth pattern (Holmes et al. 2001).

Identifying the broader causative factors will likely require inter-specific studies as the life history trait is largely fixed within species. However, Hartmann et al. (2009) have documented polymorphism for the presence/absence of telomere length change with age within the killifish (*Nothobranchius furzeri* Jubb) and Bize et al. (2009) identified all three patterns of telomere length change with age within a single population of the Alpine swift (*Apus melba* Linnaeus). These two studies show that transition between states can occur over very short evolutionary intervals and therefore the molecular and genetic control could be relatively simple.

The most obvious candidate for the control of transition between states is the ribonucleoprotein complex, telomerase, which uses an internal ribonucleic telomeric template (TERC) to elongate and maintain telomere length through the catalytic activity of the telomerase reverse transcriptase component (TERT) (Feng et al. 1995). First, while variation in the level of telomerase expression could explain the transitions in states of telomere length change with age, Elmore et al. (2008) showed that levels of telomerase expression are high in the killifish and medaka (*Oryzias latipes* Temminck & Schlegel), and yet in both species telomere length declines with age (Hayakeyama et al. 2008; Hartmann et al. 2009). Moreover, the expression of telomerase in the cell lines of birds and fish does not result generally in telomere length elongation with age or telomere length maintenance; i.e. telomere length can continue to shorten despite the expression of telomerase (e.g. Haussmann et al. 2007; Hatakeyama et al. 2008). Second, despite the constitutional expression of telomerase in the somatic tissue of the medaka, telomere length declined with age (Hayakeyama et al. 2008) and thus a simple switching between expression and non-expression would not appear to be a genetic/molecular explanation for the observed pattern of telomere length change with age. Third, the recent identification of a role for telomerase in cellular signalling, a role unrelated to telomere maintenance, may explain some of the variance in expression of telomerase (Park et al. 2009), especially if the telomere lengthening activity of telomerase is controlled to some degree by the expression of either TERC, which is encoded by a separate locus from TERT, or the numerous other proteins associated with telomere maintenance (Liu et al. 2004). Thus, variation in telomerase expression would appear an unlikely general explanation for transition between states. Despite this, telomerase has a clearly demonstrated and critical role for

telomere length maintenance and elongation and so investigations of the interactions between telomerase expression and a wider range of molecular and cellular control/regulation mechanisms will be required within species where the trait is polymorphic such as the Alpine swift.

While our macro-evolutionary analysis has identified relatively rapid evolutionary patterns of telomere length change with age in two gnathostome clades, furthering an interpretation of the biological and biochemical causes and consequences of these variable patterns will require a focus at the species level and a shift to following individuals through their lifetime. The ubiquitous distribution of the telomere structure in eukaryote chromosomes and the highly conserved telomeric sequence, contrasts with the evolutionary lability of most aspects of the biology of these fundamentally important chromosomal structures.

“I hate facts. I always say the chief end of man is to form general propositions – adding that no general proposition is worth a damn”

…Oliver Wendell Holmes (1919)

Chapter Seven

GENERAL DISCUSSION: Understanding patterns of telomere length variability: shifting our focus from the population to the individual

Accurate determinations of the age structure of aquatic vertebrate (teleost, chondrichthyan, and pinniped) populations are critical for conservation and management strategies. However, the commonly applied increment based method for ageing aquatic vertebrates suffers several limitations, foremost the general requirement to destructively sample specimens. I aimed to assess the suitability of naturally occurring changes in telomere lengths as an alternate age determinate for free-living populations of aquatic vertebrates – chondrichthyans (sharks, rays, skates, and chimaeras) [Chapter Two], teleosts (bony fishes) [Chapter Three], and pinnipeds (seals, sea lions, and walruses) [Chapter Four] and to evaluate the biological implications [Chapter Five] and patterns of evolutionary changes in this trait among jawed vertebrates (gnathostomes) [Chapter Six]. This chapter provides a General Discussion of the key findings of this thesis. For expanded discussion of the points made here and for further details, refer to the relevant text in the research chapters (Chapters Two to Six).

Telomere based organismal ageing

In species of gnathostomes, measures of telomere lengths have been shown to correlate with organismal age; and hence, telomere length measurements were suggested as a novel indicator of animal age (Haussmann & Vleck 2002). Overall, I found that telomeres do not provide an accurate means of estimating the ages of aquatic vertebrates. There was a complete lack of significant correlations between telomere length measurements and animal age, as reported for all species of chondrichthyans and approximately 75 % of the teleosts examined here, including the lungfish (Chapters Two & Five). In species where correlations between telomere length and age were identified, there was a large degree of variation between individuals associated with measurements of lengths, limiting the accuracy of age estimates based on measures of telomeres, as highlighted in the common carp (Chapter Three) and the Australian sea lion (Chapter Four). At best telomeres appeared to be limited to assigning broad age classes (Haussmann et al. 2003), or differentiating between mature and immature individuals based on measurements of telomere length (Chapter Four).

Clearly telomeres will not replace calcified structures (i.e. otoliths, vertebrae, and teeth) as a means to ageing aquatic vertebrates. Calcified structures provide a more accurate means (i.e. can be conducted with a higher degree of repeatability and precision) of estimating an animal‟s age (Campana 2001; Chapter Two) than measurements of telomere lengths. Furthermore, the enumeration of increments in calcified structures provides a more time and cost efficient ageing methodology, as well as being less technically demanding than either the terminal restriction fragment or quantitative PCR techniques used to measure telomere lengths in this thesis.

In pinnipeds the extraction of teeth (Stewart et al. 1996) and where available in fish, the use of external calcified structures (scales, fin spines and rays), provides a non-lethal (albeit invasive) means of determining the ages of free-living animals (Casselman 1983; Cailliet & Goldman 2004). However, these external calcified structures are often not available in many chondrichthyan lineages, and thus, further research is required to eliminate the need to destructively sample the animal in order to obtain an age estimate. Yet, when a fish is sacrificed in order to obtain the internal ageing structures (i.e. otoliths and vertebrate), additional biological information (i.e. state of sexual maturity, fecundity, stomach contents, parasite infection) are generally obtained in tandem to provide a composite picture of the specimen‟s condition and the state of the fish population in general. This consideration may justify the destruction of the specimen, especially when utilising fishery-dependent samples.

Variable rates of telomere length change with age

The findings of Chapters Two, Three and Four suggest that the measurement of absolute telomere length was limited in providing information on the age of individuals within a population due to the high degree of variation in telomere lengths between individuals. The variance of telomere lengths reported in between individuals may be the result of: (i) variable rates of somatic growth throughout life (Aviv et al. 2003); (ii) differences in the length of telomeres at birth (Monaghan & Haussmann 2006); (iii) marked variance in the rate of change in telomere length between individuals (Bize et al. 2009); and (iv) variable rates of exposure/resistance to “stress events” (Epel et al. 2004; Kotrschal et al. 2007). The observed variance is somewhat characteristic of the heterogeneous nature of telomeres between individuals in a typical mammalian population (Takubo et al. 2002; Monaghan & Haussmann 2006).

Alternatively, the rate of telomere length change with age appears to be a more informative measure of organismal ageing in a population (Monaghan & Haussmann 2006; Chapter Five). In birds, mammals and fish, the rate of telomere length change with age is inversely correlated with species longevity (Haussmann et al. 2003; Vleck et al. 2003; Chapter Five). The rate of telomere length change is analogous to the rate at which telomeres reach the critical length that initiates cellular senescence (Hemann et al. 2001; Haussmann et al. 2003; Vleck et al. 2003). Therefore, a better understanding of the molecular mechanisms that may contribute to variable patterns of telomere length change in fish, i.e. factors that act to restore or shorten telomeres, requires further investigation in order to elucidate the evolution of variation in species‟ longevities (Monaghan & Haussmann 2006). In addition, investigations into relationships between species‟ rates of telomere length change and growth rates, reproductive rates, and metabolic rates are required. Furthering our understanding of how these biological processes contribute to telomere length change may provide an alternate explanation as to why species have significantly different rates of telomere length change with age.

Chronological versus *physiological* age

In general, variation in telomere length between individuals appears to be characteristic of telomeric studies (Monaghan & Haussmann 2006) and the cause(s) of this variation in lengths has warranted further investigation (Aviv et al. 2003). To date, several factors have already been identified as significantly affecting an individual‟s telomere length. These include a range of genetic factors, i.e. variable telomere lengths at birth (Monaghan & Haussmann 2006) and the heritable nature of telomere length acquisition (Slagbloom et al. 1994; Njajou et al. 2007), as well as environmental factors (Goyns 2002; Hall et al. 2004), and physiological factors, i.e. diet, physical activity, perceived stress – even smoking (Jennings et al. 2000; Epel et al. 2004; Valdes et al. 2005; McGrath et al. 2007). Yet, there are potentially other equally important biological factors such as, differential growth at birth (i.e. catch up growth), the level of parental investment/care, and the onset of sexual maturity, which may also affect telomere lengths. These factors are all known to affect the rates of somatic growth in aquatic vertebrates (Helfman et al. 2009); therefore, it is likely that they will also influence telomere length change and require further investigation.

Whilst telomeres do not provide an appropriate means of determining the ages of free-living aquatic vertebrates, telomere length may be more informative as a measure of the relative fitness and condition of an individual within a population (Bize et al. 2009). Telomere measurements may be better suited as indices of animal condition, by providing a measure of

the "physiological" age of individuals, which reflects the accumulated effects of chronological ageing and stress events throughout life (Harley et al. 1992; Aviv 2002; von Zglinicki & Martin-Ruiz 2005); thus those animals with shorter telomeres in spite of their younger chronological age may be old in a biological sense (Juola et al. 2006).

The application of telomeres as a biomarker of physiological age in aquatic vertebrates requires further investigation. To adequately use telomeres as a condition index in aquatic vertebrates, a better understanding of the consequences of the observed patterns of variation in lengths between individuals is required.

Shifting focus from the population to the individual

To further our understanding of the biological and biochemical causes and consequences of variable patterns of telomere length change with age will require a shift from focusing at the population level to following individuals throughout their lifetime. To date, there are few studies where individuals have been followed throughout their lifespan (Table 1). These studies have documented that telomere length does change with age (Monaghan & Haussmann 2006). Furthermore, they have also demonstrated that following individuals through time is the best way to identify correlative links between telomere length and key biological processes, e.g. reproductive longevity/success, long-term survival, as well as somatic growth potential.

Table 1. Longitudinal studies of telomere length in gnathostomes.

Class	Species	Common name	Reference
Mammals	*Pan troglodytes*	Chimpanzee	Feng et al. (1998)
Mammals	*Felix domesticus*	Domestic cats	Brümmendorf et al. (2002)
Mammals	*Homo sapiens*	Humans	Feng et al. (1999)
Aves	*Phalacrocorax aristotelis*	Shags	Hall et al. (2004)
Aves	*Tachycineta bicolor*	Tree swallows	Haussmann et al. (2005)
Aves	*Riparia riparia*	Sand martins	Pauliny et al. (2006)
Aves	*Calidris alpina*	Dunlins	Pauliny et al. (2006)
Aves	*Apus melba*	Alpine swifts	Bize et al. (2009)
Reptile	*Liasis fuscus*	Water python	Ujvari & Madsen (2009)

Interestingly, these longitudinal studies also indicate that individuals have a characteristic mean telomere length (Takubo et al. 2002). The population level sampling of the majority of

telomeric studies potentially mask different patterns of telomere length change with age between individuals; hence, resulting in the large degree of variation in telomere length between individuals observed within a population. This is probably best highlighted by Bize et al. (2009), where different individuals of the Alpine swift (*Apus melba* Linnaeus) had one of three patterns of telomere length change with age within a single population, i.e. increasing, declining and no change in length. However, when the population of the Alpine swift was considered as a whole, there was a weak, non-significant decline in telomere length with age. More longitudinal studies are needed from a wider range of taxa that exhibit a range of life history strategies (i.e. protracted maturity, extended longevities) to sufficiently evaluate the role of telomeres in key life history processes. However, the implementation of longitudinal studies in fish would be subject to the limitations of conventional tag-recapture studies, i.e. low recapture rates of wild caught fish, the collection of tagged individuals by external parties (i.e. recreational fishers) potentially compromising DNA quality, and tag/biopsy induced mortality.

Evolutionary aspects of telomere length variability in gnathostomes

Not so surprisingly, rates of telomere length change differ among species (Monaghan & Haussmann 2006; Chapter Five), as species are subject to/undergo different degrees of environmental stress (Goyns 2002; Hall et al. 2004), rates of growth (Jennings et al. 1999) and life history patterns (Vleck et al. 2003). However, more interestingly the general pattern of telomere length change with age is also variable between species of gnathostomes – in fact this thesis contains examples of all three patterns of telomere length change with age that were outlined in the General Introduction (Chapter One): (i) no significant change in telomere length with age, as seen in the Port Jackson shark (Chapter Two); (ii) increasing telomere length with age, as reported for the common carp (Chapter Three); and (ii) declining telomere length with age, documented in the Australian sea lion (Chapter Four). Understanding the causes and consequences of these patterns have, to date, been hampered by a lack of understanding of the evolutionary history of the overall patterns of telomere length change in the gnathostome lineages.

Through ancestral state reconstruction, I was able to determine the most likely/parsimonious pattern of telomere length change with age in the common ancestor to all gnathostome lineages (Chapter 6). The ancestral state reconstruction showed that the pattern of telomere length change with age was not present ancestrally and has since evolved independently at least twice in divergent gnathostome lineages, with some secondary losses. I was also able to

map the evolutionary history of transitions to and between these three patterns of telomere length change within the available gnathostome lineages, with the birds and teleosts displaying the highest rates of evolutionary lability of patterns of telomere length change with age through repeated transitions from, and reversions to the ancestral state.

Thus, with the evolutionary origins of patterns of telomere length change with age delineated, future research can be focused to adequately address those three key questions that were highlighted in the General Introduction (Chapter One):

- What are the selective factors that constrain species in having one pattern of telomere length change with age over an alternate pattern?
- What are the selective factors that drive transitions between patterns of telomere length change with age between taxonomic units (e.g. orders, families, or species)?
- What are the benefits and, or consequences of a species possessing one of these three variable patterns of telomere length change with age as opposed to an alternate pattern?

This will be best achieved by directing the focus of future studies to those gnathostome lineages in which the transition from the ancestral state occurs repeatedly, which were identified in the Actinopterygii and bird clades (Chapter 6). Furthermore, the Actinopterygii and bird clades showed the most recent history of reversions to the ancestral state and two independent transitions to increasing telomere length with age. This will allow a shift from broad based macro-evolutionary approaches to identify the causative factors in the evolution of telomere length with age to an analysis within recently evolved groups where the transition process is still occurring and there is less impact of confounding variables, i.e. traits that vary because of phylogenetic distance.

However, the Actinopterygii and bird clades are the most widely sampled of the gnathostome lineages, with many lineages not represented. Therefore broader sampling of the other gnathostome lineages may uncover a greater degree of polymorphism of patterns of telomere length change within and between major gnathostome lineages. In addition, the inclusion of telomere length data from the hagfishes and lampreys will elucidate further the evolutionary patterns of telomere length change in the Craniata.

Identifying the broader causative factors likely will require inter-specific studies amongst closely related species as the life history trait is largely fixed within species. However,

Hartmann et al. (2009) have documented polymorphism for the presence/absence of telomere length change with age between two laboratory strains of a single species of killifish (*Nothobranchius furzeri* Jubb). This study suggests that transition between states can occur over very short evolutionary intervals and therefore the molecular and genetic control could be relatively simple.

The most obvious candidate for the control of transition between states is the ribonucleoprotein complex, telomerase, which uses an internal ribonucleic telomeric template to elongate and maintain telomere length through the catalytic activity of the telomerase reverse transcriptase component (Feng et al. 1995). In contrast to the generally observed absence of telomerase in normal somatic mammalian cell lines, telomerase has been reported at varying levels in the somatic cells of birds, reptiles and fish (Girondot & Garcia 1998; Haussmann et al. 2004, 2007; Elmore et al. 2008). However, telomeres continue to decline in length irrespective of telomerase expression (Furugori et al. 2000; Hartmann et al. 2009). Moreover, telomerase expression has been associated with other telomere independent cellular functions (Saretzki 2009), i.e. increased resilience to cellular stress (Gorbunova et al. 2002) and improve in mitochondrial function (Haendeler et al. 2009), as well as facilitating cellular signalling (Park et al. 2009) and gene expression (Saretzki 2009). Therefore, the differential expression of telomerase between species appears an unlikely universal explanation for transitions between patterns of telomere length change. Despite this, telomerase has a clearly demonstrated and critical role for telomere length maintenance and elongation and so investigations of the interactions between telomerase expression and a wider range of molecular and cellular control/regulation mechanisms are required.

Conclusion

While measurements of telomere length as an age estimator are limited by generally high degrees of variability among individuals, telomeres may alternatively provide a biomarker of an individual‟s history and condition. However, more information is required on what the causes and consequences of different patterns of telomere length change with age, which requires a change in focus to individual species‟ and a shift from the population level to following individuals throughout their lifetime. Nevertheless, telomeres may provide an immediate measure of the condition of an individual as well as a prediction of their long-term fitness.

Clearly, there is a need to broaden the taxonomic extent of telomeric research to encompass a greater diversity of life history strategies. This taxonomic expansion would be dually beneficial, by first increasing our understanding of the causes and consequences of changes in telomere length with age in species groups with vastly different life history patterns, i.e. slow versus rapid growth, protracted versus early maturity, aquatic versus terrestrial versus amphibious environs. Second, increasing the breadth of the taxonomic coverage of telomere length-at-age relationships will further enhance our understanding of the evolutionary history of telomere change in gnathostomes/vertebrates/eukaryotes.

“Telomere work is a complicated business”

…Carina Dennis (2006)

Appendix One

Supporting Material

Table A1. Sources of data for telomere length change with age in gnathostome chordates. Where state (×) indicates no significant relationship between telomere length and age; (–) telomere length decreases with age; and (+) telomere length increases with age.

Taxon	Terminal taxon name on tree	Order	State	Reference
Squalus megalops	Squaliformes	Squaliformes	×	This study
Heterodontus portusjacksoni	Heterodontiformes	Heterodontiformes	×	This study
Dentiraja lemprieri	Rajidae	Rajiformes	×	This study
Trygonorrhina dumerilii	Rhinobatidae	Rajiformes	×	This study
Urolophus paucimaculatus	Urolophidae	Rajiformes	×	This study
Callorhinchus milii	Chimaeriformes	Chimaeriformes	×	This study
Cyprinus carpio	Cypriniformes	Cypriniformes	+	This study
Thamnaconus degeni	Tetraodontiformes	Tetraodontiformes	–	This study
Platycephalus bassensis	Scorpaeniformes	Scorpaeniformes	–	This study
Dicentrarchus labrax	Moronidae	Perciformes	×	Horn et al. (2008)
Chrysophrys auratus	*Chrysophrys*	Perciformes	–	This study
Acanthopagrus schlegeli	*Acanthopagrus*	Perciformes	×	Tsui (2005)
Lutjanus argentimaculatus	Lutjanidae	Perciformes	–	Tsui (2005)
Macquaria ambigua	Percichthyidae	Perciformes	×	This study
Pseudocaranx wrighti	Carangidae	Perciformes	×	This study
Trachurus novaezelandiae	Carangidae	Perciformes	×	This study
Upeneichthys vlamingii	Mullidae	Perciformes	–	This study
Oryzias latipes	Beloniformes	Beloniformes	–	Hatakeyama et al. (2008)
Nothobranchius fasciata	Cyprinodontiformes	Cyprinodontiformes	–	Hartmann et al. (2009)
Nothobranchius rachovii		Cyprinodontiformes	–	Hsu et al. (2008)
Neoceratodus forsteri	Ceratodontiformes	Ceratodontiformes	×	This study
Xenopus laevis	Anura	Anura	×	Bassham et al. (1998)
Homo sapiens	Primates	Primate	–	Feng et al. (1998)
Pan troglodytes		Primate	–	Feng et al. (1998)
Macaca nemestrina		Primate	–	Shibata et al. (1999)
Macaca fascicularis		Primate	–	Shibata et al. (1999)
Mus spretus	Rodentia	Rodentia	–	Prowse & Greider (1995)
Ovis aries		Artiodactyla	–	Shiels et al. (1999)
Bos taurus	Artiodactyla	Artiodactyla	–	Miyashita et al. (2002)
Capra sp.		Artiodactyla	–	Betts et al. (2005)
Canis familiaris	Carnivora	Carnivora	–	Yazawa et al. (2001)
Felis catus		Carnivora	–	Brümmendorf et al. (2002)

Equus caballus	Perissodactyla	Perissodactyla	–	Katepalli et al. (2008)
Equus asinus		Perissodactyla	–	Argyle et al. (2003)
Sus scrofa		Perissodactyla	–	Kozik et al. (1998)
Emys orbicularis	Pleurodira	Chelonia	×	Girondot & Garcia (1998)
Caretta caretta	Cryptodira	Chelonia	×	Hatase et al. (2008)
Thamnophis elegans	Squamata	Squamata	–	Bronikowski (2008)
Alligator mississippiensis	Crocodilia	Crocodilia	–	Scott et al. (2006)
Gallus gallus	Galliformes	Galliformes	–	Delany et al. (2000)
Apus melba	Apodiformes	Apodiformes	×	Bize et al. (2009)
Fregata minor	Fregatidae	Pelecaniformes	–	Juola et al. (2006)
Sula nebouxii	Sulidae	Pelecaniformes	×	Foote (2008)
Phalacrocorax aristotelis	Phalacrocoracidae	Pelecaniformes	–	Hall et al. (2004)
Pygoscelis adeliae	Sphenisciformes	Sphenisciformes	–	Haussmann et al. (2003)
Oceanodroma leucorhoa	Hydrobatidae	Procellariiformes	+	Haussmann et al. (2003)
Diomedea exulans	Diomedeidae	Procellariiformes	–	Hall et al. (2004)
Macronectes giganteus	Procellariinae	Procellariiformes	–	Foote (2008)
Macronectes halli		Procellariiformes	–	Foote (2008)
Calidris alpina		Charadriformes	–	Pauliny et al. (2006)
Sterna hirundo	Charadriformes	Charadriformes	–	Haussmann et al. (2003)
Tachycineta bicolor		Passeriformes	–	Haussmann et al. (2003)
Taeniopygia guttata	Passeriformes	Passeriformes	–	Haussmann et al. (2003)
Ripria ripria		Passeriformes	–	Pauliny et al. (2006)

Table A2. GenBank accession numbers for mitochondrial nucleotide sequence data used.

Taxon	*CO1*	*cytb*	*ND4*	*12S rRNA*	*16S rRNA*
Squaliformes	NC_002012	NC_002012	NC_002012	NC_002012	NC_002012
Heterodontiformes	AJ310141	AJ310141	AJ310141	AJ310141	AJ310141
Rajidae	AY525783	AY525783	AY525783	AY525783	AY525783
Rhinobatidae	EU399098	D50023	EU795649	AF448016	EU099467
Urolophidae	EU339355	AB021501	XXXXXX	AF448028	AY836583
Chimaeriformes	AJ310140	AJ310140	AJ310140	AJ310140	AJ310140
Cypriniformes – *Cyprinus*	NC_001606	NC_001606	NC_001606	NC_001606	NC_001606
Tetraodontiformes – *Thamnaconus degeni*	AP009185	AP009185	AP009185	AP009185	AP009185
Scorpaeniformes	EF607489	NC_005450	NC_005450	NC_005450	AY539008
Moronidae	EU524145	EF427553	No data	AY372812	AB122029
Sparidae – *Chrysophrys major*	NC_003196	NC_003196	NC_003196	NC_003196	NC_003196
Sparidae – *Acanthopgarus latus*	NC_010977	NC_010977	NC_010977	NC_010977	NC_010977
Lutjanidae	NC_009869	NC_009869	NC_009869	NC_009869	NC_009869
Percichthyidae	DQ107949	AY577777	No data	AF295061	EF120874
Carangidae	AB108498	AB108498	AB108498	AB108498	AB108498
Mullidae	DQ107792	DQ197965	AP006006	FJ008147	AY947853
Beloniformes – *Oryzias*	NC_004387	NC_004387	NC_004387	NC_004387	NC_004387
Cyprinodontiformes – *Rivulus marmoratus*	AF283503	AF283503	AF283503	AF283503	AF283503
Ceratodontiformes	NC_003127	NC_003127	NC_003127	NC_003127	NC_003127
Anura – *Xenopus*	M10217	M10217	M10217	M10217	M10217
Primates – *Homo*	FJ442939	FJ442939	FJ442939	FJ442939	FJ442939
Rodentia – *Mus*	NC_006914	NC_006914	NC_006914	NC_006914	NC_006914
Artiodactyla – *Bos*	NC_006853	NC_006853	NC_006853	NC_006853	NC_006853
Carnivora – *Canis*	NC_011218	NC_011218	NC_011218	NC_011218	NC_011218
Perissodactyla – *Equus*	NC_001640	NC_001640	NC_001640	NC_001640	NC_001640
Pleurodira – *Eretmochelys imbricata*	NC_012398	NC_012398	NC_012398	NC_012398	NC_012398
Cryptodira – *Chrysemys picta*	NC_002073	NC_002073	NC_002073	NC_002073	NC_002073
Squamata – *Dinodon*	NC_001945	NC_001945	NC_001945	NC_001945	NC_001945
Crocodilia – *Alligator*	NC_001922	NC_001922	NC_001922	NC_001922	NC_001922
Galliformes – *Gallus*	NC_001323	NC_001323	NC_001323	NC_001323	NC_001323
Apodiformes – *Apus*	AM237310	AM237310	AM237310	AM237310	AM237310
Fregatidae – *Fregata*	AP009192	AP009192	AP009192	AP009192	AP009192
Sulidae	EF101675	U90006	AY567949	AF173579	L33395
Phalacrocoracidae – *Phalacrocorax*	DQ433902	EU167011	No data	EU167066	EU136707
Sphenisciformes – *Eudyptula*	NC_004538	NC_004538	NC_004538	NC_004538	NC_004538
Hydrobatidae – *Oceanodroma*	DQ433853	AF076064	AY567941	X82513	No data
Diomedeidae – *Diomedea*	NC_007172	NC_007172	NC_007172	NC_007172	NC_007172
Procellariinae – *Pterodroma*	NC_007174	NC_007174	NC_007174	NC_007174	NC_007174
Charadriformes	DQ385180	FM875773	DQ385146	DQ674571	DQ385299
Passseriformes – *Taeniopygia*	NC_007897	NC_007897	NC_007897	NC_007897	NC_007897

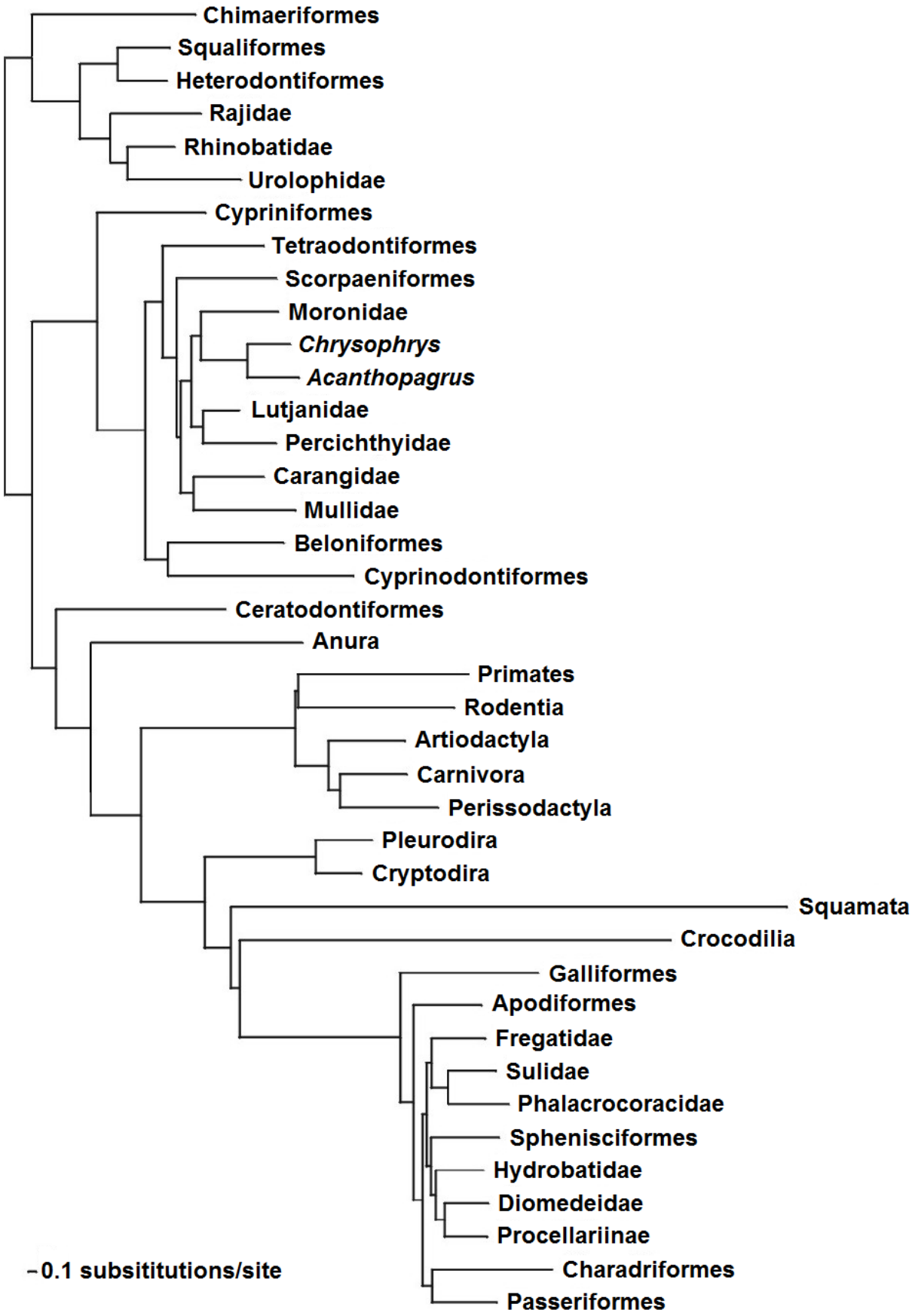

Figure A1. Phylogram of gnathostome phylogenetic relationships.

Table A3. Ancestral states inferred from maximum parsimony (MP) and maximum likelihood (ML) ancestral state reconstruction at all nodes in Figure 2, Chapter Six; where state (0) indicates no significant relationships between telomere length and age; (1) telomere length declines with age; and (2) telomere length increases with age. For ML reconstructions we present the log likelihood (logL) and proportional contributions to the total likelihood for each state (% contrib.). Note, as ML values are reported in the negative log, larger values imply lower support. * denotes the significant state.

		ML					
		State 0		State 1		State 2	
Node	MP	logL	% contrib.	logL	% contrib.	logL	% contrib.
2	0	37.1856*	0.8921*	39.5663	0.0825	40.7466	0.0253
3	0	37.1133*	0.9590*	40.5817	0.0299	41.5728	0.0111
4	0	37.0743*	0.9971*	43.3505	0.0019	43.9686	0.0010
5	0	37.0728*	0.9986*	44.3279	0.0007	44.4595	0.0006
6	0	37.0725*	0.9989*	44.5020	0.0006	44.6807	0.0005
7	0	37.0728*	0.9986*	44.3740	0.0007	44.4264	0.0006
8	0	37.2516*	0.8350*	39.0887*	0.1330*	40.5170	0.0319
9	0,1,2	38.2210	0.3168	37.7902	0.4874	38.7020	0.1958
10	1	39.9336	0.0571	37.1425*	0.9314*	41.5457	0.0114
11	1	40.0558	0.0506	37.1262*	0.9467*	42.9949	0.0027
12	1	39.8067	0.0649	37.1393*	0.9344*	44.3419	0.0007
13	0,1	39.5034	0.0879	37.1638*	0.9117*	44.9264	0.0004
14	0,1	39.1048*	0.1308*	37.2123*	0.8685*	44.6263	0.0005
15	0,1	38.6297*	0.2104*	37.3116*	0.7864*	42.8768	0.0030
16	0,1	38.4974*	0.2402*	37.3550*	0.7530*	42.0874	0.0066
17	0,1	39.0826*	0.1338*	37.2171*	0.8644*	43.4390	0.0017
18	0,1	39.2505	0.1132	37.1958*	0.8830*	42.6397	0.0038
19	1	40.3318	0.0384	37.1220*	0.9506*	41.5878	0.0109
20	0	37.2365*	0.8478*	39.1331*	0.1272*	40.7648	0.0249
21	0	37.2850*	0.8076*	38.8731*	0.1650*	40.6720	0.0273
22	0,1	37.5284*	0.63328*	38.1737*	0.3321*	40.4340	0.0346
23	1	41.3831	0.0134	37.0897*	0.9818*	42.4289	0.0047
24	1	41.4137	0.0130	37.0896*	0.9820*	42.3817	0.0049
25	1	43.6413	0.0014	37.0736*	0.9978*	44.2927	0.0007
26	1	43.9031	0.0011	37.0733*	0.9981*	44.2704	0.0007
27	0,1	37.5514*	0.61880*	38.1381*	0.3441*	40.3670	0.0370
28	0	37.0901*	0.9815*	41.3576	0.0138	42.4238	0.0047
29	1	37.7793*	0.4927*	37.8601*	0.4544*	40.0120	0.0528
30	1	37.8515*	0.4583*	37.7950*	0.4850*	39.9434	0.0566
31	1	39.9061	0.0587	37.1394*	0.9342*	42.0341	0.0070
32	1	40.0029	0.0533	37.1296*	0.9435*	42.8332	0.0031
33	1	43.9408	0.0010	37.0726*	0.9988*	46.5652	0.0001
34	1	45.5177	0.0002	37.0717*	0.9997*	48.0554	0.0000
35	1	43.7484	0.0013	37.0723*	0.9985*	45.9071	0.0001

36	1	40.4117	0.0354	37.1105*	0.9616*	42.9173	0.0029
37	1	46.3596	0.0001	37.0718*	0.9996*	45.3319	0.0003
38	1	45.5323	0.0002	37.0737*	0.9977*	43.2728	0.0020
39	1	45.8793	0.0001	37.0722*	0.9994*	44.4315	0.0006
40	1	43.9850	0.0010	37.0729*	0.9985*	44.8296	0.0004

Literature Cited

Allsopp, R. C., Chang, E., Kashefi-Aazam, M., Rogaev, E. I., Piatyszek, M. A., Shay, J. W. & Harley, C. B. (1995). Telomere shortening is associated with cell division *in vitro* and *in vivo*. *Experimental Cell Research* **220**, 194-200.

Allsopp, R. C. & Harley, C. B. (1995). Evidence for a critical telomere length in senescent human fibroblasts. *Experimental Cell Research* **219**, 130-136.

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W. & Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts. *Proceedings of the National Academy of Science, USA* **89**, 10114-10118.

Alonso, A., Martín, P., Albarrán, C., García, P., García, O., de Simón, L. F., García-Hirschfeld, J., Sancho, M., de La Rúa, C. & Fernández-Piqueras, J. (2004). Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Science International* **139**, 141-149.

Anderson, J. R., Morison, A. K. & Ray, D. J. (1992). Validation of the use of thin-sectioned otoliths for determining the age and growth of golden perch, *Macquaria ambigua* (Perciformes: Percichthyidae), in the Lower Murray-Darling Basin, Australia. *Australian Journal of Marine and Freshwater Research* **43**, 1103-1128.

Argyle, D., Ellsmore, V., E.A., G., Munro, A. F. & Nasir, L. (2003). Equine telomeres and telomerase in cellular immortalisation and ageing. *Mechanisms of Ageing and Development* **124**, 759-764.

Autexier, C. & Lue, N. F. (2006). The structure and function of telomerase reverse transcriptase. *Annual Review of Biochemistry* **75**, 493-517.

Aviv, A. (2002a). Chronology versus biology: telomeres, essential hypertension, and vascular aging. *Hypertension* **40**, 229-232.

Aviv, A. (2002b). Telomeres, sex, reactive oxygen species, and human cardiovascular aging. *Journal of Molecular Medicine* **80**, 689-695.

Aviv, A. (2008). The epidemiology of human telomeres: faults and promises. *Journals of Gerontology Series A – Biological Sciences and Medical Sciences* **63**, 979-983.

Aviv, A., Levy, D. & Mangel, M. (2003). Growth, telomere dynamics and successful and unsuccessful human aging. *Mechanisms of Ageing and Development* **124**, 829-837.

Aydos, S. E., Elhan, A. H. & Tükün, A. (2005). Is telomere length one of the determinates of reproductive life span? *Archives of Gynaecology and Obstetrics* **272**, 113-116.

Baird, D. M. (2005). New developments in telomere length analysis. *Experimental Gerontology* **40**, 363-368.

Barker, K. S., Quiniou, S. M. A., Wilson, M. R., Bengten, E., Stuge, T. B., Warr, G. W., Clem, L. W. & Miller, N. W. (2000). Telomerase expression and telomere length in immoral leukocyte lines from channel catfish *Developmental and Comparative Immunology* **24**, 583-595.

Bassham, S., Beam, A. & Shampay, J. (1998). Telomere variation in *Xenopus laevis*. *Molecular and Cellular Biology* **18**, 269-275.

Beamish, R. J. & McFarlane, G. A. (1987). Current trends in age determination methodology. In *The Age and Growth of Fish* (Summerfelt, R. C. & Hall, G. E., eds.), pp. 15-42. Iowa: The Iowa State University Press.

Beheregaray, L. B. (2008). Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Molecular Ecology* **17**, 3754-3774.

Bell, C. M., Burton, H. R. & Hindell, M. A. (1997). Growth of southern elephant seals, *Mirounga leonina*, during their first foraging trip. *Australian Journal of Zoology* **45**, 447-458.

Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., Labat, C., Bean, K. & Aviv, A. (2001). Telomere length as an indicator of biological aging: the gender effect and relation with pressure and pulse wave velocity. *Hypertension* **2**, 381-385.

Betts, D. H., Perrault, S. D., Petrik, J., Lin, L., Favetta, L. A., Keefer, C. L. & King, W. A. (2005). Telomere length analysis in goat clones and their offspring. *Molecular Reproduction and Development* **72**, 461-470.

Biessmann, H. & Mason, J. M. (1994). Telomeric repeat sequences. *Chromosoma* **103**, 154-161.

Bize, P., Criscuolo, F., Metcalfe, N. B., Nasir, L. & Monaghan, P. (2009). Telomere dynamics rather than age predict life expectancy in the wild. *Proceedings of the Royal Society of London B. Biological Science* **276**, 1679-1683.

Blackburn, E. H. (1990a). Telomeres and their synthesis. *Science* **249**, 489-490.

Blackburn, E. H. (1990b). Telomeres: structure and synthesis. *Journal of Biological Chemistry* **265**, 5919-5921.

Blackburn, E. H. (1991). Structure and function of telomeres. *Nature* **350**, 569-573.

Blackburn, E. H. (1992). Telomerase. *Annual Reviews in Biochemistry* **61**, 113-129.

Blackburn, E. H. (2000). Telomere states and cell fates. *Nature* **408**, 53-56.

Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell* **106**, 661-673.

Blasco, M. A., Gasser, S. M. & Lingner, J. (1999). Telomeres and telomerase. *Genes and Development* **13**, 2353-2359.

Bodnar, A. G., Ovellette, M., Holt, M. F. S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. & Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352.

Bone, Q., Marshall, N. B. & Blaxter, J. H. S. (1995). *Biology of Fishes*. London: Blackie Academic & Professional.

Borst, D. E., Redmond, T. M., Elser, J. E., Gonda, M. A., Wiggert, B., Chader, G. J. & Nickerson, J. M. (1989). Interphotoreceptor retinoid-binding protein. *The Journal of Biological Chemistry* **264**, 1115-1123.

Braccini, J. M., Gillanders, B. M. & Walker, T. I. (2006). Hierarchical approach to the assessment of fishing effects on non-target chondrichthyans: case study of *Squalus megalops* in southeastern Australia. *Canadian Journal of Fisheries and Aquatic Sciences* **63**, 2456-2466.

Bronikowski, A. M. (2008). The evolution of aging phenotypes in snakes: a review and synthesis with new data. *Age* **30**, 169-176.

Brown, P., Green, C., Sivakumaran, K. P., Stoessel, D. & Giles, A. (2004). Validating otolith annuli for annual age determination of common carp. *Transactions of the American Fisheries Society* **133**, 190-196.

Brown, P., Sivakumaran, K. P., Stoessel, D. & Giles, A. (2005). Population biology of carp (*Cyprinus carpio* L.) in the mid-Murray River and Barmah Forest Wetlands, Australia. *Marine and Freshwater Research* **56**, 1151-1164.

Brown, P., Sivakumaran, K. P., Stoessel, D., Giles, A., Green, C. & Walker, T. (2003). Carp population biology in Victoria. In *Marine and Freshwater Resources Institute Department of Primary Industries, 56, Snobs Creek*, p. 202.

Brownell, R. L., Jr, Tillman, M. F., di Sciara, G. N., Berggren, P. & Read, A. J. (2000). Further scrutiny of scientific whaling. *Science* **290**, 1696a.

Brümmendorf, T. M., Mak, J., Sabo, K. M., Baerlocher, G. M., Dietz, K., Abkowitz, J. L. & Lansdorp, P. M. (2002). Longitudinal studies of telomere length in feline blood cells:

implications for hematopoietic stem cell turnover in vivo. *Experimental Hematology* **30**, 1147-1152.

Bryan, T. M. & Cech, T. R. (1999). Telomerase and the maintenance of chromosome ends. *Current Opinion in Cell Biology* **11**, 318-324.

Burmeister, M., & Ulanovsky, L. (1992). *Pulsed-Field Gel Electrophoresis: Protocols, Methods, and Theories*. New Jersey: Humana Press.

Cailliet, G. M. (1990). Elasmobranch age determination and verification: an updated review. In *Elasmobranches as living resources: advances in the biology, ecology, systematics, and the status of the fisheries* (Pratt, H. L., Jr. , Gruber, S. H. & Taniuchi, T., eds.), pp. 97-109: U.S. Department of Commerce, NOAA Technical Report NMFS 90.

Cailliet, G. M., Andrews, A. H., Burton, E. J., Watters, D. L., Kline, D. E. & Ferry-Graham, L. A. (2001). Age determination and validation studies of marine fishes: do deep-dwellers live longer? *Experimental Gerontology* **36**, 739-764.

Cailliet, G. M. & Goldman, K. J. (2004). Age Determination and Validation in Chondrichthyan Fishes. In *Biology of Sharks and their Relatives* (Carrier, J., Musick, J. A. & Heithaus, M., eds.), pp. 399-439. San Diego: CRC Press.

Cailliet, G. M., Smith, W. D., Mollet, H. F. & Goldman, K. J. (2006). Age and growth studies of chondrichthyan fishes: the need for consistency in terminology, verification, validation, and growth function fitting. *Environmental Biology of Fishes* **77**, 211-228.

Callicott, R. J. & Womack, J. E. (2006). Real-time PCR assay for measurement of mouse telomeres. *Comparative Medicine* **56**, 17-22.

Camhi, M., Fowler, S. L., Musick, J. A., Bräutigam, A. & Fordham, S. V. (1998). Sharks and their Relatives - Ecology and Conservation. In *IUCN/SSC Shark Specialist Group*. IUCN, Gland, Switzerland and Cambridge, UK.

Campana, S. E. (2001). Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *Journal of Fish Biology* **59**, 197-242.

Campana, S. E. & Thorrold, S. R. (2001). Otoliths, increments, and elements: keys to a comprehensive understanding of fish populations? *Canadian Journal of Fisheries and Aquatic Sciences* **58**, 30-38.

Campbell, R. A., Gales, N. J., Lento, G. M. & Baker, C. S. (2008). Islands in the sea: extreme female natal site fidelity in the Australian sea lion, *Neophoca cinerea*. *Biology Letters* **4**, 139-142.

Campisi, J., Kim, S., Lim, C. S. & Rubio, M. (2001). Cellular senescence, cancer and aging: the telomere connection. *Experimental Gerontology* **36**, 1619-1637.

Casselman, J. M. (1983). Age and growth assessment of fish from their calcified structures - techniques and tools. *National Oceanic and Atmospheric Administration Technical Report NMFS* **8**, 1-17.

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

Cawthon, R. M., Smith, K. R., O'Brien, E., Sivatchenko, A. & Kerber, R. A. (2003). Association between telomere length in blood and mortality in people aged 60 years or older. *The Lancet* **361**, 393-395.

Cech, T. R. (2004). Beginning to understand the end of the chromosome. *Cell* **116**, 273-279.

Chang, W. Y. B. (1982). A statistical method for evaluating the reproducibility of age determination. *Australian Journal of Marine and Freshwater Research* **43**, 157-181.

Cherif, H., Tarry, J. L., Ozanne, S. E. & Hales, C. N. (2003). Aging and telomeres: a study into organ- and gender-specific telomere shortening. *Nucleic Acids Research* **31**, 1576-1583.

Chew, J. S. K., Oliveira, C., Wright, J. M. & Dobson, M. J. (2002). Molecular and cytogenetic analysis of the telomeric $(TTAGGG)_n$ repetitive sequences in the Nile tilapia, *Oreochromis niloticus* (Teleostei: Cichlidae). *Chromosoma* **111**, 45-52.

Coviello-McLaughlin, G. M. & Prowse, K. R. (1997). Telomere length regulation during postnatal development and ageing in *Mus spretus*. *Nucleic Acids Research* **25**, 3051-3058.

Criscuolo, F., Bize, P., Nasir, L., Metcalfe, N. B., Foote, C. G., Griffiths, K., Gault, E. A., & Monaghan, P. (2009). Real-time quantitative PCR assay for measurement of avian telomeres. *Journal of Avian Biology* **40**, 342-347.

de Magalhães, J. P., Costa, J. & Toussaint, O. (2005). HAGR: the Human Ageing Genomic Resources. *Nucleic Acids Research* **33**, D537-D543.

de Magalhães, J. P. & Toussaint, O. (2004). Telomeres and telomerase: a modern fountain of youth? *Rejuvenation Research* **7**, 126-133.

Delany, M. E., Krupkin, A. B. & Miller, M. M. (2000). Organization of telomere sequences in birds: evidence for arrays of extreme length and for in vivo shortening. *Cytogenetic and Cell Genetics* **90**, 139-145.

Dennis, C. (2006). A gentle way to age. *Nature* **442**, 507-508.

DFO [Canadian Department of Fisheries and Oceans] (2009). Overview of the Atlantic Seal Hunt 2006 – 2010. Version 01 September 2009. http://www.dfo-mpo.gc.ca/fm-gp/seal-phoque/reports-rapports/mgtplan-plangest0610/mgtplan-plangest0610-eng.htm

Djojosubroto, M. W., Choi, Y. S., Lee, H.-W. & Rudolph, K. L. (2003). Telomeres and telomerase in aging, regeneration and cancer. *Molecules and Cells* **15**, 164-175.

Dulvy, N. K., Ellis, J. R., Goodwin, N. B., Grant, A., Reynolds, J. D. & Jennings, S. (2004). Methods of assessing extinction risk in marine fishes *Fish and Fisheries* **5**, 255-276.

Elmore, L. W., Norris, M. W., Sircar, S., Bright, A. T., McChesney, P. A., Winn, R. N. & Holt, S. E. (2008). Upregulation of telomerase function during tissue regeneration. *Experimental Biology and Medicine* **233** 958-967.

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, USA* **101**, 17312-17315.

Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., Le, S., West, M. D., Harley, C. B., Andrews, W. H., Greider, C. W. & Villeponteau, B. (1995). The RNA component of human telomerase. *Science* **269**, 1236-1241.

Feng, Y.-R., Biggar, R. J., Gee, D., Norwood, D., Zeichner, S. L. & Dimitrov, D. S. (1999). Long-term telomere dynamics: modest increase of cell turnover in HIV-infected individuals followed for up to 14 years. *Pathobiology* **67**, 34-38

Feng, Y.-R., Norwood, D., Shibata, R., Gee, D., Xiao, X., Martin, M., Zeichner, S. L. & Dimitrov, D. S. (1998). Telomere dynamics in HIV-1 infected and uninfected chimpanzees measured by an improved method based on high-resolution two-dimensional calibration of DNA sizes. *Journal of Medical Primatology* **27**, 258-265.

Fernández-Delgado, C. (1990). Life history patterns of the common carp, *Cyprinus carpio*, in the estuary of the Guadalquivir river in south-west Spain. *Hydrobiologia* **206**, 19-28.

Fontana, F., Lanfredi, M., Chicca, M., Aiello, V. & Rossi, R. (1998). Localisation of the repetitive telomeric sequence $(TTAGGG)_n$ in four sturgeon species. *Chromosome Research* **6**, 303-306.

Foote, C. G. (2008). Avian telomere dynamics. Ph.D. Thesis. University of Glasgow, Scotland.

Francis, R. I. C. C., Paul, L. J. & Mulligan, K. P. (1992). Ageing of adult snapper (*Pagrus auratus*) from otolith annual ring counts: validation by tagging and oxytetracycline injection. *Australian Journal of Marine and Freshwater Research* **43**, 1069-1089.

Frenck, R. W., Jr. , Blackburn, E. H. & Shannon, K. M. (1998). The rate of telomere sequence loss in human leukocytes varies with age. *Proceedings of the National Academy of Sciences* **95**, 5607-5610.

Froese, R. & Pauly, D. (2008). FishBase www.fishbase.org.

Furugori, E., Hirayama, R., Nakamura, K.-I., Kammori, M., Esaki, Y. & Takubo, K. (2000). Telomere shortening in gastric carcinoma with aging despite telomerase activation. *Journal of Cancer Research and Clinical Oncology* **126**, 481-485.

Gales, N. J., Williamson, P., Higgins, L. V., Blackberry, M. A. & James, I. (1997). Evidence for a prolonged postimplantation period in the Australian sea lion (*Neophoca cinerea*). *Journal of Reproduction and Fertility* **111**, 159-163.

Garrido-Ramos, M. A., Herrán, R. d. l., Rejón, C. R. & Rejón, M. R. (1998). A satellite DNA of the Sparidae family (Pisces, Perciformes) associated with telomeric sequences. *Cytogenetic and Cell Genetics* **83**, 3-9.

Gil, M. E. & Coetzer, T. L. (2004). Real-time quantitative PCR of telomere length. *Molecular Biotechnology* **27**, 169-172.

Girondot, M. & Garcia, J. (1998). Senescence and longevity in turtles: what telomeres tell us. In *Current Studies in Herpetology* (Miaud, D. C. & Guyetant, R., eds.), pp. 133-137: Societa Europaea Herpetologica, Le Bouget du Lac, France.

Glazier, D. (2005). Beyond the "3/4-power law": variation in the intra- and interspecific scaling of metabolic rate in animals. *Biological Reviews* **80**, 611-662.

Gorbunova, V., Seluanov, A. & Pereira-Smith, O. M. (2002). Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *Journal of Biological Chemistry* **277** 38540-38549.

Gornung, E., Gabrielli, I. & Sola, A. (1998). Localization of the $(TTAGGG)_n$ telomeric sequence in zebrafish chromosomes. *Genome* **41**, 136-138.

Goyns, M. H. (2002). Genes, telomeres and mammalian ageing. *Mechanisms of Ageing and Development* **123**, 791-799.

Goyns, M. H. & Lavery, W. L. (2000). Telomerase and mammalian ageing: a critical appraisal. *Mechanisms of Ageing and Development* **114**, 69-77.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H. & de Lange, T. (1999). Mammalian telomeres end in a large duplex loop. *Cell* **97**, 503-514.

Hackett, S. J., Kimball, R. T., Reddy, S., Bowie, R. C. K., Braun, E. L., Braun, M. J., Chojnowski, J. L., Cox, W. A., Han, K.-L., Harshman, J., Huddleston, C. J., Marks, B. D., Miglia, K. J., Moore, W. S., Sheldon, F. H., Steadman, D. W., Witt, C. C. & Yuri, T. (2008). A phylogenomic study of birds reveals their evolutionary history. *Science* **320**, 1763-1768.

Haendeler, J., Dröse, S., Büchner, N., Jakob, S., Altschmied, J., Goy, C., Spyridopoulos, I., Zeiher, A. M., Brandt, U. & Dimmeler, S. (2009). Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. *Arteriosclerosis, Thrombosis, and Vascular Biology* **29**, 929-935.

Hall, M. E., Nasir, L., Daunt, F., Gault, E. A., Croxall, J. P., Wanless, S. & Monaghan, P. (2004). Telomere loss in relation to age and early environment in long-lived birds. *Proceedings of the Royal Society of London B. Biological Science* **271**, 1571-1576.

Hanna, C. W., Bretherick, K. L., Gair, J. L., Fluker, M. R., Stephenson, M. D. & Robinson, W. P. (2009). Telomere length and reproductive aging. *Human Reproduction* **1**, 1-6.

Harley, C. B., Futcher, A. B., & Greider, C. W. (1990). Telomeres shorten during aging of human fibroblasts. *Nature* **345**, 458-460.

Harley, C. B., Vaziri, H., Counter, C. M. & Allsopp, R. C. (1992). The telomere hypothesis of cellular aging. *Experimental Gerontology* **27**, 375-382.

Hartmann, N., Reichwald, K., Lechel, A., Graf, M., Kirschner, J., Dorn, A., Terzibasi, E., Wellner, J., Platzer, M., Rudolph, K. L., Cellerino, A. & Englert, C. (2009). Telomeres shorten while Tert expression increases during ageing of the short-lived fish *Nothobranchius furzeri*. *Mechanisms of Ageing and Development* **130**, 290-296.

Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K. & Allshire, R. C. (1990). Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**, 866-868.

Hatakeyama, H., Nakamura, K.-I., Izumiyama-Shimomura, N., Ishii, A., Tsuchida, S., Takubo, K. & Ishikawa, N. (2008). The teleost *Oryzias latipes* shows telomere shortening with age despite considerable telomerase activity throughout life. *Mechanisms of Ageing and Development* **129**, 550-557.

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

Haussmann, M. F. & Mauck, R. A. (2008a). New strategies for telomere-based age estimation. *Molecular Ecology Notes* **8**, 264-274.

Haussmann, M. F. & Mauck, R. A. (2008b). Telomeres and longevity: testing an evolutionary hypothesis. *Molecular Biology and Evolution* **25**, 220-228.

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

Haussmann, M. F., Vleck, C. M. & Nisbet, I. C. T. (2003a). Calibrating the telomere clock in common terns, *Sterna hirundo*. *Experimental Gerontology* **38**, 787-790.

Haussmann, M. F., Winkler, D. W., Huntington, C. E., Nisbet, I. C. T. & Vleck, C. M. (2004). Telomerase expression is differentially regulated in birds of differing life span. *Annual New York Academy of Science* **1019**, 186-190.

Haussmann, M. F., Winkler, D. W., Nisbet, I. C. T., Huntington, C. E. & Vleck, C. M. (2007). Telomerase activity is maintained throughout the lifespan of long-lived birds. *Experimental Gerontology* **42**, 610-618.

Haussmann, M. F., Winkler, D. W., O'Reilly, K. M., Huntington, C. E., Nisbet, I. C. T. & Vleck, C. M. (2003b). Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings of the Royal Society of London. Series B: Biological Science* **270**, 1387-1392.

Haussmann, M. F., Winkler, D. W. & Vleck, C. M. (2005). Longer telomeres associated with higher survival in birds. *Biology Letters* **1**, 212-214.

Helfman, G. S., Collette, B. B., Facey, D. E. & Bowen, B. W. (2009). *The Diversity of Fishes. Second edition.* Oxford, UK: Wiley-Blackwell.

Hemann, M. T., Strong, M. A., Hao, L. Y. & Greider, C. W. (2001). The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* **107**, 67-77.

Higgins, L. V. (1993). The nonannual, nonseasonal breeding cycle of the Australian sea lion, *Neophoca cinerea*. *Journal of Mammalogy* **74**, 270-274.

Hoenig, J. M. & Gruber, S. H. (1990). Life history patterns in elasmobranches: implications for fisheries management. In *Elasmobranches as living resources: advances in the biology, ecology, systematics, and the status of the fisheries* (Pratt, H. L., Jr., Gruber, S. H. & Taniuchi, T., eds.), pp. 1-15: U.S. Department of Commerce, NOAA Technical Report NMFS 90.

Holmes, D., Flückiger, R. & Austad, S. N. (2001). Comparative biology of aging in birds: an update. *Experimental Gerontology* **36**, 869-883.

Horn, P. L. (1993). Growth, age structure, and productivity of jack mackerels (*Trachurus* spp.) in New Zealand waters. *New Zealand Journal of Marine and Freshwater Research* **27**, 145-155.

Horn, T., Gemmell, N. J., Robertson, B. C. & Bridges, C. R. (2008). Telomere length change in European sea bass (*Dicentrarchus labrax*). *Australian Journal of Zoology* **56**, 207-210.

Hsu, C.-Y., Chiu, Y.-C., Hsu, W.-L. & Chan, Y.-P. (2008). Age-related markers assayed at different developmental stages of the annual fish *Nothobranchius rachovii*. *Journal of Gerontology: Biological Sciences* **63A**, 1267-1276.

Huffman, K. E., Levene, S. D., Tesmer, V. M., Shay, J. W. & Wright, W. E. (2000). Telomere shortening is proportional to the size of the G-rich telomeric-3‟-overhang. *Journal of Biological Chemistry* **275**, 19719-19722.

Hugall, A. F., Foster, R. & Lee, M. S. Y. (2007). Calibration choice, rate smoothing, and the pattern of tetrapod diversification according to the long nuclear gene RAG-1. *Systematic Biology* **56**, 543-563.

Izzo, C. (2005). Aging Port Jackson Sharks, *Heterodontus portusjacksoni* (Meyer 1793), using calcified structures. Honours thesis. Flinders University, Australia.

Izzo, C. & Gillanders, B. M. (2008). Initial assessment of age, growth and reproductive parameters of the southern fiddler ray *Trygonorrhina fasciata* (Müller & Henle, 1841) from South Australia. *Pan-American Journal of Aquatic Sciences* **3**, 321-327.

Janvier, P. (1997). Gnathostomata. Jawed Vertebrates. Version 01 January 1997 (under construction). http://tolweb.org/Gnathostomata/14843/1997.01.01 ***In:*** *The Tree of Life Web Project*, http://tolweb.org/

Jeanclos, E., Schork, N. J., Kyvik, K. O., Kimura, M., H.Skurnick, J. & Aviv, A. (2000). Telomere length inversely correlates with pulse pressure and is highly familial. *Hypertension* **36**, 195-200.

Jemielity, S., Kimura, M., Parker, K. M., Parker, J. D., Cao, X., Aviv, A. & Keller, L. (2007). Short telomeres in short-lived males: what are the molecular and evolutionary causes? *Aging Cell* **6**, 225-233.

Jennings, B. J., Ozanne, S. E., Dorling, M. W. & Hales, C. N. (1999). Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *Federation of European Biochemical Societies: Letters* **448**, 4-8.

Jennings, B. J., Ozanne, S. E. & Hales, C. N. (2000). Nutrition, oxidative damage, telomere shortening, and cellular senescence: individual or connected agents of aging? *Molecular Genetics and Metabolism* **71**, 32-42.

Joeng, K. S., Song, E. J., Lee, K. J. & Lee, J. (2004). Long lifespan in worms with long telomeric DNA. *Nature Genetics* **36**, 607-611.

Juola, F. A., Haussmann, M. F., Dearborn, D. C. & Vleck, C. M. (2006). Telomere shortening in a long-lived marine bird: cross-sectional analysis and test of an ageing tool. *The Auk* **123**, 775–783.

Katepalli, M. P., Adams, A. A., Lear, T. L. & Horohov, D. W. (2007). The effect of age and telomere length on immune function in the horse *Developmental and Comparative Immunology* **32**, 1409-1415.

Keefe, D. L. & Liu, L. (2009). Telomeres and reproductive aging. *Reproduction, Fertility and Development* **21**, 10-14.

King, J. R. & McFarlane, G. A. (2003). Marine fish life history strategies: applications to fishery management. *Fisheries Management and Ecology* **10**, 249-264.

Kishi, S., Uchiyama, J., Baughman, A. M., Goto, T., Lin, M. C. & Tsai, S. B. (2003). The zebrafish as a vertebrate model of functional aging and very gradual senescence. *Experimental Gerontology* **38**, 777-786.

Koehn, J. D. (2004). Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater Biology* **49**, 882-894.

Koopman, M. & Morison, A. K. (2002). Sand flathead – a bread and butter catch. *Marine and Freshwater Resources Institute: Research and Education*, 1.

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

Kozik, A., Bradbury, E. M. & Zalensky, A. (1998). Increased telomere size in sperm cells of mammals with long terminal $(TTAGGG)_n$ arrays *Molecular Reproduction and Development* **51**, 98-104.

Lahnert, P. (2005). An improved method for determining telomere length and its use in assessing age in blood and saliva. *Gerontology* **51**, 352-356.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. & Higgins, D. G. (2007). ClustalW2 and ClustalX version 2. *Bioinformatics* **23**, 2947-2948.

Last, P. R. & Stevens, J. D. (2009). *Sharks and rays of Australia. Second Edition.* Collingwood, VIC: CSIRO Publishing.

Lee, H.-W., Blasco, M. A., Gottlieb, G. J., Horner, J. W., Greider, C. W. & DePinho, R. A. (1998). Essential role of mouse telomerase in highly proliferative organs. *Nature* **392**, 569-574.

Levy, M. Z., Allsopp, R. C., Futcher, A. B., Greider, C. W. & Harley, C. B. (1992). Telomere end-replication problem and cell aging. *Journal of Molecular Biology* **225**, 951-960.

Lin, K. W. & Yan, J. (2005). The telomere length dynamic and methods of its assessment. *Journal of Cellular and Molecular Medicine* **9**, 977-989.

Ling, J. K. (1992). *Neophoca cinerea*. *Mammalian Species* **393**, 1-7.

Ling, J. K., Atkin, C., Barnes, A., Fischer, A., Guy, M. & Pickering, S. (2006). Breeding and longevity in captive Australian sea lions *Neophoca cinerea* at zoos and aquaria in Australia: 1965-2003. *Australian Mammalogy* **28**, 65-76.

Lingner, J. & Cech, T. R. (1998). Telomerase and chromosome end maintenance. *Current Opinion in Genetics and Development* **8**, 226-232.

Liu, D., O'Connor, M. S., Qin, J. & Songyang, Z. (2004). Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *Journal of Biological Chemistry* **279**, 51338-51342.

López, J. A., Ryburn, J. A., Fedrigo, O. & Naylor, G. J. P. (2006). Phylogeny of sharks of the family Triakidae (Carcharhiniformes) and its implications for the evolution of carcharhiniform placental viviparity. *Molecular Phylogenetics and Evolution* **40** 50-60.

Lund, T. C., Glass, T. J., Tolar, J. & Blazar, B. R. (2009). Expression of telomerase and telomere length are unaffected by either age or limb regeneration in *Danio rerio*. *PLoS ONE* **4**, e7688. doi:10.1371/journal.pone.0007688

Maddison, W. P. & Maddison, D. R. (2009). Mesquite: a modular system for evolutionary analysis. Version 2.01, http://mesquiteproject.org.

Mangel, M. & Abrahams, M. (2001). Age and longevity in fish, with consideration of the ferox trout. *Experimental Gerontology* **36**, 765-790.

McGrath, M., Wong, J. Y. Y., Michaud, D., Hunter, D. J. & Vivo, I. D. (2007). Telomere length, cigarette smoking, and bladder cancer risk in men and women. *Cancer Epidemiology Biomarkers & Prevention* **16**, 815-819.

McIntosh, R. R. (2007). Life history and population demographics of the Australian sea lion. In *Department of Zoology, School of Life Sciences, Faculty of Science, Technology and Engineering*. La Trobe University: Bundoora, Victoria.

McKevitt, T. P., Nasir, L., Wallis, C. V. & Argyle, D. J. (2003). A cohort study of telomere and telomerase biology in cats. *American Journal of Veterinary Research* **64**, 1496-1499.

McKevitt, T. P., Nasir, L., Devlin, P. & Argyle, D. J. (2002). Telomere lengths in dogs decrease with increasing donor age. *Journal of Nutrition* **132**, 1604s-1606s.

McNeil, N. & Reid, T. (2000). Novel molecular cytogenetic techniques for identifying complex chromosomal rearrangements: technology and applications in molecular medicine. *Expert Reviews in Molecular Medicine* **2000**, 1-14.

Mefford, H. C. & Trask, B. J. (2002). The complex structure and dynamic evolution of human subtelomeres. *Nature Review Genetics* **3**, 91-102.

Metcalf, S. J. & Swearer, S. E. (2005). Non-destructive ageing in *Notolabrus tetricus* using dorsal spines with an emphasis on the benefits for protected, endangered and fished species. *Journal of Fish Biology* **66**, 1740-1747.

Meyne, J., Baker, R. J., Hobart, H. H., Hsu, T. C., Ryder, O. A., Ward, O. G., Wiley, J. E., Wurster-Hill, D. H., Yates, T. L. & Moyzis, R. K. (1990). Distribution of non-telomeric sites of the (TTAGGG)n telomeric sequence in vertebrate chromosomes. *Chromosoma* **99**, 3-10.

Meyne, J., Ratliff, R. L. & Moyzis, R. K. (1989). Conservation of the human telomere sequence $(TTAGGG)_n$ among vertebrates. *Proceedings of the National Academy of Science, USA* **86**, 7049-7053.

Miyashita, N., Shiga, K., Yonai, M., Kaneyama, K., Kobayashi, S., Kojima, T., Goto, Y., Kishi, M., Aso, H., Suzuki, T., Sakaguchi, M. & Nagai, T. (2002). Remarkable differences in telomere lengths among cloned cattle derived from different cell types. *Biology of Reproduction* **66**, 1649-1655.

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

Moyzis, R. K., Buckingham, J. M., Cram, L. S., Dani, M., Deaven, L. L., Jones, M. D., Meyne, J., Ratliff, R. L. & Wu, J.-R. (1988). A highly conserved DNA sequence $(TTAGGG)_n$ present at the telomeres of human chromosomes. *Proceedings of the National Academy of Science, USA* **85**, 6622-6626.

Murnane, J., Sabatier, L., Marder, B. & Morgan, W. (1994). Telomere dynamics in an immortal human cell line. *European Molecular Biology Organization Journal* **13**, 4953-4962.

Musick, J. A. (1999). Ecology and conservation of long-lived marine animals. In *Life in the slow lane: Ecology and conservation of long-lived marine animals* (Musick, J. A., ed.), pp. 1-10. Bethesda, Massachusetts: American Fisheries Society Symposium 23.

Nakagawa, S., Gemmell, N. J. & Burke, T. (2004). Measuring vertebrate telomeres: applications and limitations. *Molecular Ecology* **13**, 2523-2533.

Nasir, L., Devlin, P., McKevitt, T., Rutteman, G. & Argyle, D. J. (2001). Telomere lengths and telomerase activity in dog tissues: a potential model system to study human telomere and telomerase biology. *Neoplasia* **3**, 351-359.

Nelson, J. S. (2006). *Fishes of the world. 4th edition*. New Jersey: John Wiley and Sons.

Neumann, A. & Reddel, R. (2002). Telomere maintenance and cancer - look, no telomerase. *Nature Reviews Cancer* **2**, 879-884.

Nikolskii, G. V. (1969). *Theory of Fish Population Dynamics as the Biological Background for Rational Exploitation and Management of Fishery Resources*. Edinburgh: Oliver and Boyd.

Njajou, O. T., Cawthon, R. M., Damcott, C. M., Wu, S.-H., Ott, S., Garant, M. J., Blackburn, E. H., Mitchell, B. D., Shuldiner, A. R. & Hsueh, W.-C. (2007). Telomere length is paternally inherited and is associated with parental lifespan. *Proceedings of the National Academy of Science, USA* **104**, 12135-12139.

Nordgård, O., Kvaloy, J. T., Farmen, R. K., & Heikkilä, R. (2006). Error propagation in relative real-time reverse transcription polymerase chain reaction quantification models: The balance between accuracy and precision. *Annals of Biochemistry* **356**, 182-193.

Nosek, J., Tomáška, L. & Kucejová, B. (2004). The chromosome end replication: lessons from mitochondrial genetics. *Journal of Applied Biomedicine* **2**, 71-79.

Nunn, G. B. & Stanley, S. E. (1998). Body size effects and rates of cytochrome b evolution in tube-nosed seabirds. *Molecular Biology and Evolution* **15**, 1360-1371.

O‟Callaghan, N. J., Dhillon, V. S., Thomas, P. & Fenech, M. (2008). A quantitative real-time PCR method for absolute telomere length. *BioTechniques* **44**, 807-809.

Ohsumi, S. (1995). The necessity of employing lethal methods in the study of whale resources. In *Research on Whales*, p. 38. Tokyo, Japan: The Institute of Cetacean Research.

Okuda, K., Bardeguez, A., Gardner, J. P., Rodriguez, P., Ganesh, V., Kimura, M., Skurnick, J., Awad, G., & Aviv, A. (2002). Telomere length in the newborn. *Paediatric Research* **52**, 377-381.

Olovnikov, A. M. (1973). A theory of marginotomy: The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *Journal of Theoretical Biology* **41**, 181-190.

Pagnozzi, J. M., Silva, M. J. d. J. & Yonenaga-Yassuda, Y. (2000). Intraspecific variation in the distribution of the interstitial telomeric (TTAGGG)n sequences in *Micoureus demerarae* (Marsupialia: Didelphidae). *Chromosome Research* **8**, 585-591.

Pardue, M.-L. & DeBaryshe, P. G. (1999). Telomeres and telomerase: more than the end of the line. *Chromosoma* **108**, 73-82.

Park, J.-I., Venteicher, A. S., Hong, J. Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P., Ji, H., McLaughlin, M., Veenstra, T. D., Nusse, R., McCrea, P. D. & Artandi, S. E. (2009). Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature* **460**, 66-72.

Pauliny, A., Wagner, R. H., Augustin, J., Szép, T. & Blomqvist, D. (2006). Age-independent telomere length predicts fitness in two bird species. *Molecular Ecology* **15**, 1681-1687.

Pauly, D. (1987). Application of information on age and growth of fish to fishery management. In *The Age and Growth of Fish* (Summerfelt, R. C. & Bonfil, R., eds.), pp. 495-506. Iowa: The Iowa State University Press.

Perez, J., Moran, P. & Garcia-Vazquez, E. (1999). Cloning and physical mapping of Atlantic salmon (*Salmo salar* L.) telomeric sequences. *Heredity*, 409-414.

Platell, M. E. & Potter, I. C. (2001). Partitioning of food resources amongst 18 abundant benthic carnivorous fish species in marine waters on the lower west coast of Australia. *Journal of Experimental Marine Biology and Ecology* **261**, 31-54.

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

Quinn, T. J. & Dersio, R. B. (1999). *Quantitative Fish Dynamics*. New York, New York: Oxford University Press.

Ratnasingham, S. & Hebert, P. D. N. (2007). BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**, 355-364.

Reddel, R. R. (2003). Alternative lengthening of telomeres, telomerase, and cancer. *Cancer Letters* **194**, 155–162.

Reif, W. (1978). Wound healing in sharks: form arrangement of repair scales. *Zoomorphologie* **90**, 101-111.

Rocco, L., Costagliola, D. & Stingo, V. (2001). $(TTAGGG)_n$ telomeric sequence in selachian chromosomes. *Heredity* **87**, 583-588.

Rocco, L., Stingo, V. & Bellitti, M. (1996). Cloning and characterization of a repetitive DNA detected by HindIII in the genome of *Raja montagui* (Batoidea, Chondrichthyes). *Gene* **176**, 185-189.

Rose, M. R. (1991). *Evolutionary biology of aging*. New York: Oxford Press.

Rufer, N., Brümmendorf, T. H., Kolvraa, S., Bischoff, C., Christensen, K., Wadsworth, L., Schulzer, M. & Lansdorp, P. M. (1999). Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood *Journal of Experimental Medicine* **190**, 157-167.

Ruiz-Herrera, A., Nergadze, S. G., Santagostino, M., & Giulotto, E. (2008). Telomeric repeats far from the ends: mechanisms of origin and role in evolution. *Cytogenetics and Genome Research* **122**, 219–228.

Saldanha, S. N., Andrews, L. G. & Tollefsbol, T. O. (2003). Assessment of telomere length and factors that contribute to its stability. *European Journal of Biochemistry* **270**, 389-403.

Salvadori, S., Deiana, A., Elisabetta, C., Floridia, G., Rossi, E. & Zuffardi, O. (1995). Colocalization of (TTAGGG)n telomeric sequences and ribosomal genes in Atlantic eels. *Chromosome Research* **3**, 54-58.

Saretzki, G. (2009). Telomerase, mitochondria and oxidative stress. *Experimental Gerontology* **44**, 485-492.

Sato, J. J., Hosoda, T., Wolsan, M., Tsuchiya, K., Yamamoto, M. & Suzuki, H. (2003). Phylogenetic relationships and divergence times among Mustelids (Mammalia: Carnivora) based on nucleotide sequences of the nuclear interphotoreceptor retinoid binding protein and mitochondrial cytochrome b genes. *Zoological Science* **20**, 243-264.

Sato, J. J., Wolsan, M., Suzuki, H., Hosoda, T., Yamaguchi, Y., Hiyama, K., Kobayashi, M. & Minami, S. (2006). Evidence from nuclear DNA sequences sheds light on the phylogenetic relationships of Pinnipedia: single origin with affinity to Musteloidea. *Zoological Science* **23**, 125-146.

Schluter, S. F. & Marchalonis, J. J. (2003). Cloning of shark RAG2 and characterization of the RAG1/RAG2 gene locus. *The Federation of American Societies for Experimental Biology Journal.* **17**, 470-472.

Schwartz, F. J. & Maddock, M. B. (2002). Cytogenetics of the elasmobranchs: genome evolution and phylogenetic implications. *Marine and Freshwater Research* **53**, 491–502

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

Shibata, R. R., Feng, Y. R., Gee, D., Norwood, D., Xiao, X., Zeichner, S. L., Martin, M. A. & Dimitrov, D. S. (1999). Telomere dynamics in monkeys: increased cell turnover in macaques infected with chimeric simian-human immunodeficiency viruses. *Journal of Medical Primatology* **28**, 1-10.

Shiels, P. G., Kind, A. J., Campbell, K. H. S., Waddington, D., Wilmut, I., Colman, A. & Schnieke, A. E. (1999). Analysis of telomere lengths in cloned sheep. *Nature* **399**, 316-317.

Sigma Aldrich (2008). qPCR Technical Guide. p. 40: Sigma Aldrich.

Slagboom, P. E., Droog, S. & Boomsma, D. I. (1994). Genetic determination of telomere size in humans: a twin study of three age groups. *The American Journal of Human Genetics* **55**, 866-869.

Smith, B. B. (2005). The state of the art: a synopsis of information on common carp (*Cyprinus carpio*) in Australia. In *Final Technical Report, SARDI Aquatic Sciences Publication No. RD04/0064-2; SARDI Research Report Series No. 77, prepared by the South Australian Research and Development Institute (Aquatic Sciences)*, p. 68. Adelaide.

Southern, E. M. & Elder, J. K. (1995). Theories of gel electrophoresis of high molecular weight DNA. In *Pulsed Field Gel Electrophoresis – A Practical Approach*. (Monaco, A. D. ed). pp. 1-21. Oxford: Oxford University Press.

Stamatakis, A. (2006). RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688-2690.

Stearns, S. C. (1992). *The evolution of life histories*. New York: Oxford University Press.

Stewart, R. E., Stewart, B. E., Stirling, I. & Street, E. (1996). Counts of growth layer groups in cementum and dentine in ringed seals (*Phoca hispida*). *Marine Mammal Science* **12**, 383-401.

Svane, I., Rodda, K. & Thomas, P. (2007). Prawn fishery by-catch and discards: marine ecosystem analysis - population effects. *Final Technical Report, SARDI Aquatic Sciences Publication No. RD03-0132. SARDI Research Report Series No. 199, prepared by the South Australian Research and Development Institute (Aquatic Sciences)*, Adelaide.

Swango, K. L., Timken, M. D., Chong, M. D. & Buoncristiani, M. R. (2006). A quantitative PCR assay for the assessment of DNA degradation in forensic samples. *Forensic Science International* **158**, 14-26.

Takubo, K., Izumiyama, S., N., Honma, N., Sawabe, M., Arai, T., Kato, M., Oshimura, M. & Nakamura, K. I. (2002). Telomere lengths are characteristic in each human individual. *Experimental Gerontology* **37**, 523-531.

Thomas, P., O" Callaghan, N. J. & Fenech, M. (2008). Telomere length in white blood cells, buccal cells and brain tissue and its variation with ageing and Alzheimer"s disease. *Mechanisms of Ageing and Development* **129**, 183-190.

Thorson, T. B., Langhammer, J. K. & Oetinger, M. I. (1988). Periodic shedding and replacement of venomous caudal spines, with special reference to South American freshwater stingrays, *Potamotrygon* spp. *Environmental Biology of Fishes* **23**, 299-314.

Tovar-Ávila, J., Izzo, C., Walker, T. I., Braccini, J. M. & Day, R. W. (2008). Dorsal-fin spine growth of *Heterodontus portusjacksoni*: a general model that applies to dorsal-fin spines of chondrichthyans? *Canadian Journal of Fisheries and Aquatic Sciences* **65**, 74-82.

Tovar-Ávila, J., Walker, T. I. & Day, R. W. (2007). Reproduction of *Heterodontus portusjacksoni* in Victoria, Australia: evidence of two populations and reproductive parameters for the eastern population. *Marine and Freshwater Research* **58**, 956-965.

Tsui, J. C. Y. (2005). Evaluation of telomere length as an age-marker in marine teleosts. Ph.D. thesis. The University of Hong Kong.

Tsuji, A., Ishiko, A., Takasaki, T. & Ikeda, N. (2002). Estimating age of humans based on telomere shortening. *Forensic Science International* **125**, 197-199.

Ujvari, B. & Madsen, T. (2009). Short telomeres in hatchling snakes: erythrocyte telomere dynamics and longevity in tropical pythons. *PLoS ONE* **4,** e7493. doi:10.1371/journal.pone.0007493

Valdes, A. M., Andrew, T., Gardner, J. P., Kimura, M., Oelsner, E., Cherkas, L. F., Aviv, A. & Spector, T. D. (2005). Obesity, cigarette smoking, and telomere length in women. *The Lancet* **366**, 662-664.

Valdesalici, S. & Cellerino, A. (2003). Extremely short lifespan in the annual fish *Nothobranchius furzeri*. *Proceedings of the Royal Society of London. Series B, Biological Sciences* **270** (Suppl 2), S189-S191.

Vaziri, H., Schachter, F., Uchida, I., Zhu, L. W. X., Effros, R., Cohen, D. & Harley, C. B. (1993). Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *American Journal of Human Genetics* **52**, 661-667.

Venkatesan, R. N. & Price, C. (1998). Telomerase expression in chickens: constitutive activity in somatic tissues and down-regulation in culture. *Proceedings of the National Academy of Sciences* **95**, 14763-14768.

Venkatesh, B., Erdmann, M. V. & Brenner, S. (2001). Molecular synapomorphies resolve evolutionary relationships of extant jawed vertebrates. *Proceedings of the National Academy of Science, USA* **98**, 11382-11387.

Vilizzi, L. & Walker, K. F. (1999). Age and growth of the common carp, *Cyprinus carpio*, in the River Murray, Australia; validation, consistency of age interpretation and growth models. *Environmental Biology of Fishes* **54**, 77-106.

Vleck, C. M., Haussmann, M. F. & Vleck, D. (2003). The natural history of telomeres: tools for aging animals and exploring the aging process. *Experimental Gerontology* **38**, 791-796.

von Zglinicki, T. (2002). Oxidative stress shortens telomeres. *Trends in Biochemical Sciences* **27**, 339-344.

von Zglinicki, T. (2003). Replicative senescence and the art of counting. *Experimental Gerontology* **38**, 1259-1264.

von Zglinicki, T. & Martin-Ruiz, C. M. (2005). Telomeres as biomarkers for ageing and age-related diseases. *Current Molecular Medicine* **5**, 197-203.

von Zglinicki, T., Pilger, R. & Sitte, N. (2000). Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radical Biology & Medicine* **28**, 64-74.

Walker, T. I. (2004). Management Measures. In *Elasmobranch Fisheries Management Techniques* (Musick, J. A. & Bonfil, R., eds.), pp. 285-323. Singapore: APEC.

White, W. T. & Potter, I. C. (2005). Reproductive biology, size and age compositions and growth of the batoid *Urolophus paucimacalatus*, including comparisons with other species of the Urolophidae. *Marine and Freshwater Research* **56**, 101-110.

Yazawa, M., Okuda, M., Setoguchi, A., Iwabuchi, S., Nishimura, R., Sasaki, N., Masuda, K., Ohno, K. & Tsujimoto, H. (2001). Telomere length and telomerase activity in canine mammary gland tumors. *American Journal of Veterinary Research* **62**, 1539-1543.

Yoda, M., Takahashi, K. G. & Mori, K. (2002). Telomerase activity detected in eyed embryos of rainbow trout *Oncorhynchus mykiss*. *Fisheries Science* **68**, 132-137.

Zakian, V. A. (1989). Structure and function of telomeres. *Annual Reviews of Genetics* **23**, 579-604.

Zambrano, L., Martínez-Meyer, E., Menezes, N. & Peterson, A. T. (2006). Invasive potential of common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) in American freshwater systems. *Canadian Journal of Fisheries and Aquatic Sciences* **63**, 1903-1910.

Zeichner, S. L., Palumbo, P., Feng, Y. R., Xiao, X., Gee, D., Sleasman, J., Goodenow, M., Biggar, R. & Dimitrov, D. (1999). Rapid telomere shortening in children. *Blood* **93**, 2824-2830.