Development of a DNA based aging technique for use in fisheries assessments

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Contents

Acronyms and Abbreviations

AFLP .....Amplified fragment length polymorphism
ANOVA .....Analysis of variance
AVG .....Abalone Viral Ganglioneuritis
bp.....Base pairs
CCD .....Charge-coupled device
CV .....Coefficient of variation
DEEDI .....Department of Employment, Economic Development and Innovation (Queensland)
DIG.....Digoxigenin
DNA .....Deoxyribonucleic acid
DTT .....Dithiothreitol
EDTA.....Ethylenediaminetetraacetic acid
FIGE.....Fluctuating inverted field gel electrophoresis
FISH .....Fluorescence *in situ* hybridisation
FRDC .....Fisheries Research & Development Corporation
FTE .....Full-time equivalent
hrs.....Hours
IST.....Interstital and sub-telomeric
kb .....Kilobase pairs
LSD .....Least significant difference
mins.....Minutes
mL.....Millilitre
MW.....Molecular weight (in base pairs)
NSW.....New South Wales
Q-PCR.....Quantitative polymerase chain reaction
QLD.....Queensland
ROS .....Reactive oxygen species
SD .....Standard Deviation
SDS.....Sodium Dodecyl Sulphate
secs .....Seconds
TAE .....Tris acetate EDTA buffer
TL.....Telomere length
TRAP.....Telomere repeat amplification protocol
TRF .....Terminal restriction fragment

Non-Technical Summary

2007/033 Development of a DNA based aging technique for use in fisheries assessments

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OBJECTIVES:

1. To evaluate a variety of laboratory methods for estimating telomere length.
2. To characterise the relationship between age and telomere length in a species that is most likely to yield positive results.
3. If Objective 2 is achieved, the project will characterise the relationship between age and telomere length in other species and test the robustness of the relationships to external variables.
4. To evaluate the usefulness of the relationship between age and telomere length for selected species in terms of the requirements for fisheries stock assessment.

OUTCOMES ACHIEVED TO DATE:

1. Telomere length (DNA biomarker for age) was measured with accuracy and precision in a range of invertebrate fisheries species in relation to variables such as age (or size), gender, tissue type and environment.
2. A relationship between shell size and telomere length was demonstrated for wild-caught, black-lip abalone (*Haliotis rubra*).
3. Telomere length is likely to be more accurate for determining age in molluscan rather than crustacean species.
4. Further research is recommended in the following areas:
   a. Telomere length in relation to age (as opposed to shell size) in *Haliotis rubra*.
   b. Telomere length in relation to age and body condition in *Haliotis rubra* and other commercially important mollusc species (e.g. *Pecten fumatus*).
   c. A PCR-based method of assaying telomere length, which would allow the analysis of large numbers of individuals accurately, rapidly and more cheaply than the method used here (TRF assay).

SUMMARY:

The productivity of a fisheries resource can be quantified from estimates of recruitment, individual growth and natural and fisheries-related mortality, assuming the spatial extent of the resource has been quantified and there is minimal immigration or emigration. The sustainability of a fisheries resource is facilitated by management controls such as minimum and maximum size limits and total allowable catch. Minimum size limits are often set to allow individuals the opportunity to reproduce at least once before the chance of capture. Total allowable catches are a proportion of the population biomass, which is estimated based on known reproduction, recruitment, mortality and growth rates.

In some fisheries, however, management actions are put in place without quantification of the resource through the stock assessment process. This occurs because species-specific information, for example individual growth, may not be available. In these circumstances, management actions need to be precautionary to protect against future resource collapse, but this often means that the resource is lightly exploited. Consequently, the productivity of the resource is not fully realised.

Australia's most valuable fisheries are invertebrate fisheries (Australian Department of Agriculture Fisheries and Forestry, 2008). For example, Australian fisheries (i.e. excluding aquaculture) production of crustaceans (largely prawns, rock lobster and crab) was 41,000 tonnes in 2006/7, worth $778 million. Production from mollusc (largely abalone, scallops, oysters and squid) fisheries was 39,000 tonnes, worth $502 million. Together, in 2006/7 crustacean and mollusc fisheries represented 58% of the total value of Australian wild fisheries production. Sustainable management of Australia's invertebrate fisheries is frustrated by the lack of data on species-specific growth rates.

This project investigated a new method to estimate age, and hence individual growth rates, in invertebrate fisheries species. The principle behind the new aging method was that telomeres (i.e. DNA end-caps of chromosomes) get shorter as an individual gets older.

We studied commercial crustacean and molluscan species. A vertebrate fish species (silver perch, *Bidyanus bidyanus*) was used as a control to standardise our work against the literature. We found a clear relationship between telomere length and shell size for temperate abalone (*Haliotis rubra*). Further research is recommended before the method can be implemented to assist management of wild-harvested abalone populations. Age needs to be substituted for shell size in the relationship and it needs to be studied for abalone from several regions. This project showed that telomere length declined with increasing age in Sydney rock oysters (*Saccostrea glomerata*) and was affected by regional variation.

A relationship was not apparent between telomere length and age (or size as a surrogate for age) for crustacean species (school prawns, *Metapenaeus macleayi*; eastern rock lobster, *Sagmariasus verreauxi*; southern rock lobster, *Jasus edwardsii*; and spanner crabs, *Ranina ranina*). For school prawns, there was no difference between telomere length in males and females. Further research is recommended, however, as telomeric DNA from crustaceans was difficult to analyse using the terminal restriction fragment (TRF) assay. Telomere lengths of spanner crabs and lobsters were at the upper limit of resolution of the assay used and results were affected by degradation and possible contamination of telomeric DNA.

It is possible that telomere length is an indicator of remaining lifespan in molluscan and crustacean individuals, as suggested for some vertebrate species (e.g. Monaghan, 2010). Among abalone of similar shell size and among lobster pueruli, there was evidence of individuals having significantly longer or shorter telomeres than the group average. At a population level, this may be a surrogate for estimates of future natural mortality, which may have usefulness in the management of those populations.

The method used to assay telomere length (terminal restriction fragment assay) performed adequately for most species, but it was too expensive and time-consuming to be considered a useful tool for gathering information for fisheries management. Research on alternative methods is strongly recommended.

KEYWORDS:

Telomere, age and growth, terminal restriction fragment assay, Sydney rock oysters, abalone, spanner crabs, school prawns, rock lobster, silver perch, fisheries stock assessment.

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1. Background

Knowledge of individual growth rates and the age structure of fisheries populations are crucial for accurate population modeling, which is an integral component of the management cycle. For example, age data for species such as abalone and spanner crabs would decrease the degree of uncertainty in stock assessment models and allow managers to maximise the economic use of the resource while remaining within sustainable harvest limits. The importance of aging spanner crabs was highlighted by Brown and others (1999, 2008). Management of other invertebrate species, such as rock lobster, abalone and beche-de-mer, would also benefit from age information.

Teleosts and Elasmobranchii species have hard structures, such as otoliths or vertebrae, which can be examined for evidence of incremental growth and hence age (Campana, 2001). Using hard structures to calculate growth is lethal, which is not ideal if the individual has high conservation importance. Growth, if not age, can be estimated by mark recapture methods where tagged fish are released and recaptured after a known time at liberty. The tagging process requires extensive fieldwork, and thus is expensive and time consuming. Estimates depend on recapturing tagged animals and in many studies the frequency of recaptures is low (Stevick *et al.* 2001; Sumpton *et al.* 2003).

Age and growth information of mollusc species has commonly been obtained using tag-recapture methods (McShane *et al.* 1988), counting growth rings of shells or by studying the ratio of isotopes in the carbonate of shells (Goewert and Surge, 2008; Gurney *et al.* 2005; Keller *et al.* 2002; Naylor *et al.* 2007) and cohort or length frequency analyses (Shepherd *et al.* 1995; Siddeek and Johnson 1997; Tarr, 1995). Although these methods have provided useful information for some species, they can be time consuming, labour intensive, require careful validation and the process of tagging or sampling can be deleterious or lethal to the organism.

Crustaceans cannot be aged with methods used for finfish or molluscs. The accumulation of the pigment lipofuscin is used as an index of crustacean age (Sheehy, 1990), however lipofuscin assays are expensive and time-consuming. Neural tissue containing pigment needs to be dissected and the amount of pigment present determined by histology, histofluorimetry or chemical assay. The method is lethal and requires specialist equipment and expertise.

Telomeres are structures at the ends of linear chromosomes composed of short tandem highly repeated DNA sequences and associated proteins. The main function of telomeres is to protect the ends of the chromosomes from degradation and provide genome stability. Haussmann and Vleck (2002) and others suggested that telomeric DNA at the ends of chromosomes could provide a tool for aging animals since telomeric DNA normally shortens with progressive cell divisions over an animal's life-span. A significant correlation has been observed between age and telomere length for humans and some species of birds (Haussmann and Vleck, 2002; Haussmann *et al.* 2003; Vleck *et al.* 2003) and fish (Hatakeyama *et al.* 2008a; Hatakeyama *et al.* 2008b; Nakagawa *et al.* 2004; Ying, 2005).

The ability to estimate the age of a fish or crustacean from a DNA sample would be a significant addition to the valuable data that can be obtained from DNA for fisheries species. For example, DNA can be used as a replacement for plastic tags (Buckworth *et al.* 2007), as a surrogate for spawning population size (Ovenden *et al.* 2007), as a marker for stock structure (Broderick *et al.* in press; Ovenden *et al.* 2009; Sumpton *et al.* 2008) and to determine gender (McHale *et al.* 2008).

2. Need

An aging technique that is quick, reliable, accurate, cost effective and non-lethal is highly desirable, particularly for invertebrate species. This project explored an aging technique that is based on telomeric DNA. As each cell duplicates itself during the normal tissue repair process, telomeric DNA gets shorter. Thus, telomere length could be used as a surrogate to age individuals without a known age history.

This project trials a new methodology for measuring telomere DNA and relating it to age. It is relevant to (1) invertebrate fisheries, where there is no straightforward method for measuring individual growth rates, (2) teleost fisheries, where it may augment otolith aging methods, and (3) species of high conservation status, where the animal cannot be sacrificed.

Accurately aging and evaluating fish populations was important in the FRDC Research, Development and Extension Plan (2005-2010) under strategy challenge #1 ‘Natural Resource Sustainability’. The Queensland Fishing Industry Research Advisory Committee and Northern Territory Fisheries Advisory Board both regard the development of innovative methods for stock assessment as an important component of their research and development strategy.

This project was also designed around state and federal government research and development plans. Queensland’s Research and Development Priorities include ‘Enabling Technologies’, which specifies the use of biotechnology to improve the sustainability and economic viability of regional and rural communities, and ‘Environmentally Sustainable Queensland’, goals of which include support for research that facilitates the ecologically sustainable development of coastal zones. The Australian Government’s National Research priority goals include the sustainable use of Australia’s biodiversity that promotes the more comprehensive understanding of natural systems.

3. Methods

3.1 Species selection

The project team and steering committee developed criteria for the selection of model species for the project. Generally, two life stages (cohorts) per species were sampled that represented old (i.e. large) and young (i.e. small) animals. Where the age of the cohorts was unknown, size was used as a surrogate for age.

The project tested if there was a significant difference between telomere lengths in the two cohorts sampled per species. If there was, then further age (or size) cohorts were sampled from that species and the relationship between age and telomere length was investigated in further detail. The effect of variables such as tissue type and environment on the relationship in these species was investigated where possible.

In the first instance, the selection of species to test was guided by the following criteria (not necessarily in this order):

1. Invertebrate species were chosen over vertebrates.
2. We preferred short-lived species, as they could be produced in aquaculture for specific experiments within the project, if required. However, long-lived species were also desirable to provide contrast to short-lived species.
3. We focussed on species that had plastic growth characteristics. A reliable aging method was more important for these species, where size is a poor surrogate for age. Crustaceans are renowned for plastic growth rates.
4. Species were selected from a range of environments (e.g. marine to freshwater), to test the wide occurrence of the relationship.
5. Same-age or same-size samples needed to be feasibly accessed from aquaculture farms or fisheries to avoid the expense of producing samples specifically for the project.
6. Spanner crabs and school prawns should be among the first species tested, as these species had been the subjects of pilot studies.
7. The value of the commercial fishery for the species was important, with those of higher value preferred.
8. Samples should represent a range of major taxa.

The issue of calibrating the rate of telomere attrition in wild-caught samples using captive-bred samples was acknowledged to be a real challenge for all species apart from fish, which may be aged with some degree of accuracy from wild-caught material. Long-lived, slow growing species are less suitable candidates for aquaculture, but they are also difficult to age and in many cases the need for age data (e.g. age at recruitment, longevity, reproductive lifespan, etc.) to assist in sustainable management is high. If a relationship was found for a species where known age animals could not be obtained (e.g. spanner crabs), this would need to be addressed in future research.

Members of the steering committee were asked to use the criteria to rank species (Table 1). The following groups were chosen for experiments (in this order).

1. **Crustaceans:** school prawns (*Metapenaeus macleayi*, Haswell 1879), spanner crabs (*Ranina ranina*, Linneaus 1758), red rock lobsters (*Jasus edwardsii*, Hutton 1875) and green rock lobsters (*Sagmariasus verreauxi*, H. Milne Edwards 1851);
2. **Molluscs:** oysters (*Saccostrea glomerata*, Gould 1850) and abalone (*Haliotis rubra*); and
3. **Fish:** silver perch (*Bidyanus bidyanus*, Mitchell 1838).

Table 1: Summary of ranking of species for the experimental program (10 being 'high priority', to 0 being 'low priority') by members of the steering committee (SM – Steve Montgomery, PG – Phil Gaffney, SF – Stewart Frusher, IB – Ian Brown) based on tissues available to 5 June 2008.

Taxonomic Group	Species	Sample details	Known age (A) or size (S)?	Value of commercial fishery	Short (S) or Long (L) lived?	No. of cohorts	Approx. sample size/cohort	Rank			
								SM	PG	SF	IB
Mollusc	Sydney rock oyster *S. glomerata*	40 oysters approx. 3 yrs old, 35 large (4 yrs olds), 27 small (1 yr old) Four tissue types taken.	A	Low	S	2	35	9	8	10	10
Crustacean	Spanner crab *R. ranina*	Sampled 130 wild crabs in late March. 50 of each size class: small, medium and large.	S	High	?	3	50	10	7	5	8
Crustacean	Mud crab *S. serrata*	30 from feed trials. All approx. 4 months old. Crab carapace length was recorded, and muscle, hepatopancreas and gills kept for all samples.	A	High	S	1	30	0	6	3	4
Crustacean	Mud crab *S. serrata*	Three tissues from each animal (brain, hepatopancreas, muscle) in ethanol. Eleven age classes (sample size): 83 days (6), 116 days (6), 143 days (6), 170 days (6), 206 days (9), 207 days (5), 241 days (6), 262 (6), 360 (6), 366 (8), 402 (6). Sexes known.	A/S	High	S	11	6	6	8	5	8
Crustacean	Green rock lobster *S. verreauxi*	Ten large lobsters from Wallis Lakes Co-Op. Steve Montgomery has 2 yo and 1 yo juveniles (100 of each).	A/S	High	L	2	100	7	-	-	7
Crustacean	Red rock lobster *J. edwardsii*	31 puerulus samples and 42 older individuals (age unknown), muscle and digestive gland samples.	S/A	High	L	2	35	10	6	10	6
Crustacean	School prawn *M. macleayi*	Wild, from otter trawlers, Grafton, Clarence River. 150 individuals sampled. Three size classes: 25 males and 25 females in each size class. Muscle tissue only collected.	S	Medium	S	3	50	10	4	5	8

Taxonomic Group	Species	Sample details	Known age (A) or size (S)?	Value of commercial fishery	Short (S) or Long (L) lived?	No. of cohorts	Approx. sample size/cohort	Rank			
								SM	PG	SF	IB
Crustacean	Banana prawn *F. merguiensis*	52 banana prawns approx. 2 mths old. Older samples to come. Known-age animals sampled at end of April, when they were 7 months old. Culture to be harvested at 9 months.	A	High	S	2, but will have 3	50	10	3	7	5
Crustacean	Tiger prawn *P. monodon*	80 individuals young, very small, placed in tubes, and 35 in individual alfoil packages.	A	High	S	1	35	2	3	0	0
Crustacean	Red claw *C. quadricarin-atus*	53 individuals approx. 2½ months old were sampled and muscle tissue from each stored.	A/S	Low	S??	1, but will have 2 or 3	50	5	3	0	5
Fish	Whiting *S. cillata*	20 whiting approx. 60 days old, whole bodies placed in tubes, older size classes to be sampled at about 9 months of age.	A	Low	S	Will have 3	20	7	7	0	5
Fish	Silver perch *B. bidyanus*	Three age classes: 3 month old, 1 year old and 2 years old sampled from freshwater hatchery, Grafton, NSW. 30 individuals sampled from each size class. Wherever possible, heart, muscle and liver tissues were taken.	A	Low?	S	3	30	8	6	8	7

3.2 Estimating the relationship between telomere length and age

3.2.1 Outline of telomere length estimation method

The procedure for estimating telomere lengths in the laboratory in relation to age was divided into five major steps:

1. Sampling and storage of fresh tissues. A number of different tissues were obtained which may differ in their mitotic activity and thereby differ in telomere length and attrition rate.
2. Extraction of total genomic DNA from these tissues.
3. Measurement of telomere length assessed by the standard Terminal Restriction Fragment (TRF) assay, which utilises Southern hybridisation.
    a. Digestion of genomic DNA by restriction endonucleases;
    b. Separation of digested DNA by gel electrophoresis;
    c. Transfer of DNA to nylon membranes; and
    d. Chemiluminescence and detection.
4. Calculation of mean telomere length for each sample from resulting autoradiographs.
5. Statistical analyses. Detailed statistical analysis is not presented in this report. For further details refer to scientific manuscripts provided in Appendix 5.

The following sections provide details of these experimental procedures.

3.2.2 Sampling and storage of tissues

Brief details on samples used for experiments are described below. Other samples were collected, but were not used in this project (Appendix 4).

During the project, and for the foreseeable future, samples are stored at the Queensland Agricultural Biotechnology Centre, The University of Queensland, St. Lucia, in liquid nitrogen or at -80°C.

Further details on samples included in experiments are presented in the scientific manuscripts (Appendix 5).

3.2.2.1 Molluscs

Sydney rock oyster *(Saccostrea glomerata)*

Fresh Sydney rock oysters were obtained from an oyster farm in Moreton Bay, southeast Queensland and another farm in Wallis Lake, Tuncurry, New South Wales (NSW). Moreton Bay oysters were sampled in 2007 and 2009. Both locations provided approximately 25 individuals from each of three known age classes (i.e. one, three and four years of age).

Live oysters were opened and mantle, gill and adductor tissue were dissected from all age classes except the one-year-old NSW oysters, from which the whole body was removed. The tissues were immediately placed in tubes (Cyrotubes, Nunc brand, Thermo Fisher Inc., Rochester, NY, USA) and snap-frozen in liquid nitrogen. The tubes were subsequently transferred to a freezer (-80°C) for long-term storage.

Abalone (*Haliotis* spp.)

Tropical abalone (*H. asinina*) of two age classes (11 and 18 months old) were obtained and muscle tissue from the foot was sampled, frozen and stored as above. These animals were grown in aquaculture at the Bribie Island Research Centre, Queensland.

Hybrid abalones (*H. rubra* x *H. laevigata*) were obtained from a commercial aquaculture facility in Tasmania. There were three age classes in the hybrid species: four years old (64-73 mm shell), three years old (42-50 mm shell) and two years old (18-25 mm shell). Muscle tissue was sampled, frozen and stored as above.

Wild caught *H. rubra* were obtained from a naturally occurring population at George III Rock Research Area, southern Tasmania, with shell sizes ranging 20-155 mm. Muscle tissue was sampled, frozen and stored as above.

3.2.2.2 Crustaceans

Priority crustacean species of known age were not available, therefore different sized animals were used instead.

School prawn (*Metapenaeus macleayi*)

Small and medium sized prawns were caught in Wooloweyah Lagoon (NSW) and large ocean prawns were caught off shore near Yamba (northeast coast of NSW). Only muscle tissue was sampled from school prawns. Fifty individuals were taken in each size class, with half being female and half being male. Tissues were sampled, frozen and stored as above.

Attempts were made to obtain school prawns of a known age from commercial or government hatcheries. Although previously a species produced in aquaculture, when we made extensive enquiries in December 2009, it appeared that commercial prawn farms were no longer producing this species. Potential broodstock was collected from the Maroochy River (southeast Queensland) in March 2010 for the production of school prawns at the Bribie Island Research Centre. Spawning was achieved in captivity, however the eggs were unfertilised. This may have been due to immaturity of the broodstock (individuals were small), unequal sex ratios (only eight of about 60 were male) or spawning behaviour (females do not store sperm). Timely replacement of broodstock was not possible.

Eastern rock lobster (*Sagmariasus verreauxi*)

Wild caught large (average weight approximately 1 kg) and very large (average weight greater than 1.8 kg) eastern rock lobsters were obtained from commercial operators from the mid New South Wales coast. Three-year-old and puerulus lobsters were obtained from Industry & Investment NSW, Cronulla Fisheries Research Centre. These samples were wild caught and reared in captivity. A range of tissues (i.e. muscle, gill and heart) were sampled, frozen and stored as above.

Southern rock lobster (*Jasus edwardsii*)

Adult *J. edwarsdsii* were obtained from the wild from the Sandstone Bluff area, Tasmania (latitude 42°S 20’16”S, longitude 148°E 13’0”). Pueruli (31) were caught from the wild in Tasmanian waters, but their exact origin was unknown. Muscle and digestive gland tissue was sampled, frozen and stored as above.

Spanner crab (*Ranina ranina*)

Three size classes (small, medium and large) of 80-140 mm carapace width were obtained from a southeast Queensland fishery near Caloundra. Forty samples were collected per size class. Muscle, gill and eye-stalk tissue was sampled, frozen and stored as above.

3.2.3 Extraction of total genomic DNA

Total genomic DNA was extracted from frozen tissue using refinements of the standard method (Sambrook and Russell, 2001).

Tissues were taken from the freezer (-80°C) and partly thawed, and approximately 100 mg of fresh tissue was removed. The remainder was immediately re-frozen.

1. 100 mg of tissue was ground to a fine powder in liquid N_2 using a mortar and pestle, then added to 1 mL of SDS/Proteinase K digestion buffer (10 mM Tris pH 8.0, 5 mM EDTA 0.5%SDS) in a 15 mL Falcon™ tube and mixed gently.
2. 2 µL RNase (100 mg/mL) and Proteinase K to a final concentration of 200 µg/mL were then added and the contents of the tubes mixed gently.
3. The tubes were then incubated for 3-5 hours at 50°C or overnight at 37°C. Often lysis and Proteinase K digestion were performed at 50°C but it has been suggested that the lower incubation temperature reduces oxidative damage of the telomeric DNA (Lu *et al.* 2004).
4. Following SDS/ProK lysis, a 1/10 volume of 5 M potassium acetate was added to each tube and mixed well by inverting. The tubes were incubated on ice for thirty minutes.
5. Precipitated protein material was collected by centrifuging at 10,000 rpm in a fixed angle rotor for ten minutes.
6. The supernatant containing the DNA was carefully removed to a fresh tube and the precipitated protein pellet discarded.
7. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was then added to the DNA and mixed by inverting the tubes gently about six times.
8. The phases were separated by centrifugation in swing out bucket rotor at 5,000 rpm for five minutes.
9. The upper aqueous phase containing the DNA was removed to a fresh tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently by inverting the tubes about six times.
10. Phases were again separated by centrifugation as in Step 8 and the upper aqueous phase containing the DNA transferred to a fresh tube.
11. DNA was precipitated by adding two volumes of ice-cold absolute ethanol to each tube, mixing by inversion and storing at -20°C for at least ten minutes.
12. The precipitated DNA was then collected by centrifuging the tubes at 5,000 rpm in swing-out bucket rotor for 25 minutes.
13. The ethanol was carefully poured off and the DNA pellet washed with 1 mL of 70% ethanol.
14. DNA was again pelleted by centrifuging at 5,000 rpm in a swing-out bucket rotor for 10-15 minutes.
15. The ethanol was carefully discarded without dislodging the DNA pellet and the pellet left to air dry for approximately ten minutes.
16. The resulting DNA was then redissolved in 50-100 µL TE buffer pH 8.0. Dithiothreitol (DTT) was added to a final concentration of 1 mM to minimise oxidation and the samples stored at 4°C.

3.2.4 TRF (terminal restriction fragment) assays

TRF assays provide a measure of the mean telomere length across all chromosomes in cells from a given tissue sample. TRF assays were conducted on genomic DNA extracted from a range of tissue types per animal. For instance, oyster TRF assays were performed on mantle, gill, adductor muscle or whole animal.

A single TRF experiment generally consisted of 18 samples per assay, such as six individuals for three tissue types or six individuals for three age classes.

The TRF assays were performed using the TeloTTAGGG kit (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW, Australia) with the following specifications and modifications. Two micrograms of genomic DNA was digested with restriction enzymes *Hinf*I and *Rsa*I for three hours at 37°C and separated using conventional gel electrophoresis on a 1 x TAE 0.8% agarose gel at 35 V for 20 hours. Samples were loaded in a randomised order to minimise within gel effects and each TRF assay was repeated on at least two and usually three separate occasions.

The transfer, hybridisation, chemiluminescence and detection were as per the manufacturer's directions. Chemiluminescent signals from the membrane were captured on x-ray film using multiple exposures ranging from twenty seconds to thirty minutes as appropriate.

Mean telomere length (TL) was calculated by quantitative analysis of chemiluminescent signals. X-ray film can measure light over a linear dynamic range of 1:100, therefore it was necessary to obtain an exposure which was within the linear range of detection in order to make valid estimate of intensity. Overexposure of the film leads to a decrease in sensitivity and a reduction in the ability to quantify telomeres of different lengths. Correct exposure time was determined experimentally (see below).

3.2.5 Data analysis

Mean telomere length (TL) was obtained by comparing the telomere smear to a molecular size standard. X-Ray films (i.e. autoradiographs) were scanned using a high definition scanner and uploaded into Quantity One v 4.2.3 imaging software (Biorad, Gladesville, NSW, Australia) for analysis.

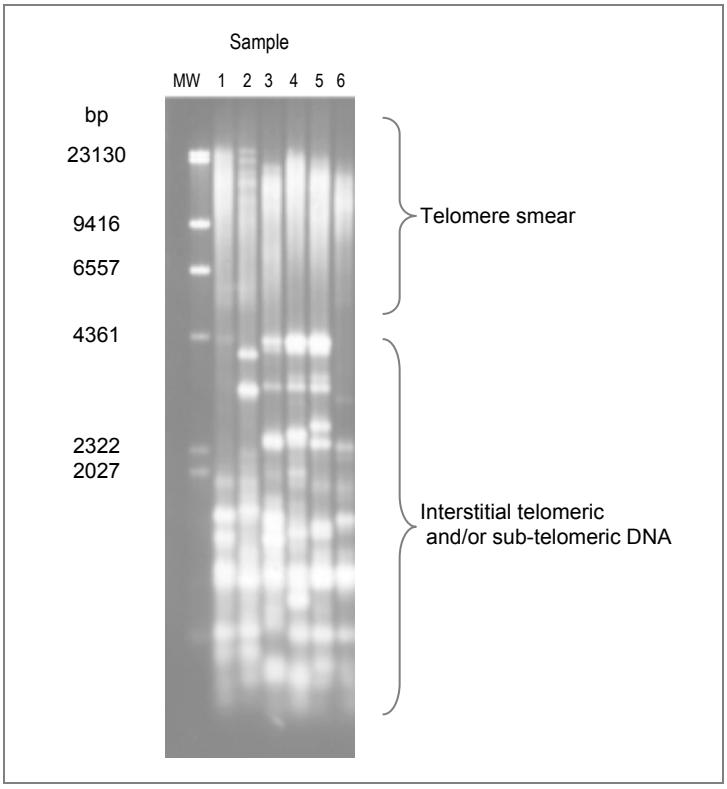

Figure 1: Oyster samples from mantle DNA were assayed for mean telomere length using the TRF assay. Telomeric DNA appears on the x-ray film as a smear above a prominent banding pattern resulting from either interstitial telomeric and sub-telomeric DNA. Each lane was overlaid with a grid of 25 boxes and total intensity and molecular weight were estimated for each box.

An example of an autoradiograph obtained from an oyster TRF assay is shown in Figure 1. The telomere smear is visible in the top half of each lane and below each smear is a distinctive polymorphic banding pattern that was characteristic of oysters. We believe this banding pattern is associated with interstitial (in the middle of chromosomes, not at the end) telomeric DNA or incomplete telomere repeats found in the subtelomeric (adjacent to telomeres) region. Interstitial telomeric DNA consists of large blocks of (TTAGGG)n repeats localised at intra-chromosomal sites, while sub-telomeric regions are adjacent to the true telomeres and are composed of several hundred base pairs of repeats with many intervening degenerate units (Lin and Yan, 2008).

Mean TL measurements were determined by the method outlined in the Roche TeloTTAGG Telomere Length Assay Protocol. In summary, each lane was overlaid with a grid of 25 boxes, which in the case of oysters, spanned the lane from the top molecular weight marker to just above the bands associated with the interstitial telomeric and subtelomeric (Figure 1).

At least two boxes were positioned above the top of the smear and all boxes were of an identical size. The total intensity data inside in each box and molecular weight at the centre of each box was estimated using Quantity One software.

A minimum background subtraction as suggested in the Roche protocol was applied in each lane whereby the box with the lowest density was subtracted from each of the remaining 24 boxes in each lane. Background subtraction removes the extra fluorescence (i.e. density) from the telomere DNA smear that may be due to undigested DNA that has randomly hybridised to the probe.

Mean Telomere Length was calculated using the formula

$$\text{Mean TRF} = \Sigma (ODi)/ \Sigma (ODi/Li)$$

where OD is the total chemiluminescent signal in each box and Li is the length of the TRF fragment at the position i. This formula takes into account the higher signal intensity from larger TRF fragments due to multiple hybridisations of the telomere specific hybridisation probe.

Two background subtraction methods (i.e. other than minimum background substraction) were explored. These were:

1. Global background subtraction: a background box was defined in an area outside the lanes and the total intensity in this box was subtracted from all other boxes on the autoradiograph; and
2. Adjacent background subtraction: a mathematical algorithm was used where background values were obtained using a linear formula calculated between the lowest intensity (background) box at the upper end of each lane and the lowest intensity (background) box at the bottom of the lane (Harley *et al.* 1999).

Following evaluation (see below), these two background subtraction methods were not adopted. The first method made little allowance for variations caused by differences in the amount of DNA loaded per lane and the second was found to be too subjective.

An analysis of variance (ANOVA) was conducted on the resulting TL measurements in base pairs (bp) using GenStat [v 11.1 (PC/Windows) 2009, 15:51:07, Copyright 2008, VSN International Ltd.]. The individual animals were taken as the independent experimental units, with split-plots adopted for the different tissues (when appropriate) and also for the replicate measurements of the same individuals. Telomere length was the dependent variable, with age groups and tissue types being the independent factors for these analyses. Each telomere length dataset was checked for normality and homogeneity of residual variances. If these underlying assumptions were met, the standard error of the difference of the means (based on pooled variances) and Fisher's least significant difference (LSD) at the 5% level was presented. If datasets were not normally distributed, they were evaluated and subjected to appropriate transformations on an *ad hoc* basis before analysis.

We used Fisher's LSD to perform pair-wise comparisons of treatment group means after a significant ANOVA was observed. LSDs are the standard errors of the differences between the means multiplied by the *t*-statistic for the residual degrees of freedom.

Estimates of between-experiment and within-experiment variation were made by running the same sample three times on a single gel and by repeating samples across at least three gels. The coefficient of variation (CV) for each source of error was calculated using the following formula

$$\% \text{ CV} = \sigma/\mu \times 100$$

where σ is the standard deviation and μ is the mean of the repeated measurements.

3.2.6 Testing the effect of environment on telomere attrition rate

The relationship between telomere length (TL) and size or age of selected species (e.g. oysters) was determined from a range of habitats along the east coast of Australia (i.e. from north to south). This was used to test the expectation that the rate of telomere attrition was constant across habitats.

3.3 Taxon-specific modifications to methods

3.3.1 Molluscs

The telomere-repeat sequence of many molluscs has been reported to be identical to the TTAGGG telomere sequence of vertebrates (Estabrooks, 1999; Guo and Allen, 1997; Plohl *et al.* 2002; Sakai *et al.* 2005). This was confirmed for Sydney rock oysters by dot blot and the commercial kit (i.e. from Roche) was used without modification of the DNA probe for oysters and abalone.

3.3.2 Crustaceans

A telomere-repeat sequence of TTAGG (i.e. distinct from the molluscs and vertebrates TTAGGG) has been reported for a number of crustacean species such as freshwater shrimp (Sahara *et al.* 1999), planktonic crustaceans and fish lice (Vitkova *et al.* 2005) and lobsters, *Homarus americanus* (Klapper *et al.* 1998).

This sequence was confirmed here for both school prawns and eastern rock lobsters by dot blot. Therefore, a $(TTAGG)_6$ oligonucleotide probe with a 3' digoxigenin (DIG) label was commercially synthesised (Sigma Genosys Pty. Ltd., Castle Hill, NSW, Australia) and used in TRF assays for crustacean telomere DNA.

Crustacean (i.e. eastern and southern rock lobster, school prawn and spanner crab) genomic DNA was more resistant to restriction enzyme digestion than vertebrate or mollusc genomic DNA. The TRF assay method relies on the digestion of genomic DNA that leaves the telomeric DNA intact. We tested various combinations of restriction enzymes to successfully digest crustacean DNA.

The restriction endonucleases required to digest (i.e. remove) the non-telomeric genomic DNA of crustaceans was tested experimentally. Preliminary experiments revealed that digesting lobster DNA with *Hin*fI and *Rsa*I achieved only partial digestion. Other frequently cutting restriction endonucleases, such as *Aci*I, *Hpy*CH4V, *Mse*I, *Taq*I, *Tsp*5091 and *Alu*I, were tested in combination on lobster DNA. The most complete digestion was obtained using *Alu*I and *Hpy*CH4V. However, for school prawns, *Rsa*I and *Hin*fI were found to be the best combination of enzymes to use for digestions. Therefore, for each species, two micrograms of genomic DNA was digested with the appropriate enzyme combination for three to five hrs at 50°C.

School prawn and southern rock lobster telomeres were separated using conventional gel electrophoresis on a 1 x TAE 0.8% agarose gel at 35V for 20 hours. However, eastern rock lobster telomeres were found to beyond the limit of resolution of conventional electrophoresis (i.e. too large) and a Fluctuating Inverted Field Gel Electrophoresis (FIGE) apparatus was employed. Several FIGE programs were tested to optimise conditions and the best separation was found to occur using a program optimised for separation of fragments 25-50 kb (forward voltage 180V, reverse voltage 120V, switch time 0.4-0.8 linear shape, run time 20 hours). Eastern rock lobster DNA was separated using these conditions on a 0.5 x TBE, 1% pulsed-field agarose gel (Biorad Cat Number 162-0137). Samples were loaded in a randomised order to minimise within gel effects and TRF assays were repeated on at least two and usually three separate occasions. A higher range molecular weight DNA size marker was used, which allowed more accurate determination of the size of the DNA fragments up to 50 kb.

Separated crustacean DNA fragments were transferred to a positively charged nylon membrane as per the procedure outlined in the TeloTTAGG kit (Roche). The hybridisation was also similar except that 10 pmol of $(TTAGG)_6$ crustacean probe (as described above) per mL Dig EasyHyb was used instead of

the probe which came with the kit. The hybridisation was performed at 50°C and the high stringency wash was conducted at 55°C to improve the specificity of the hybridisation. Chemiluminescence and detection were as per the manufacturer's directions and the signals were captured on x-ray film as described for above.

4. Results and Discussion

4.1 Optimisation of TRF analyses

There was no consensus in the literature about TRF methods for the estimation of telomere length, and published results are difficult to interpret and standardise. As TRF was the primary method for measuring telomere length in this project, and differences between age classes may be small, quantification of these errors and the adoption of a standard measurement technique were considered critical components of this project.

Telomere length can vary as a result of different measurement methodologies and the experimental error associated with each. The main sources of TL measurement error are:

- Inevitable variation during the running of the multi-day TRF assay associated with the dynamics of DNA digestion, electrophoresis, chemiluminescence and detection and preparation of the autoradiograph (particularly exposure time); and
- Inconsistencies of the measurement of TRF on the autoradiograph such as drawing and positioning intensity boxes on the scan and the method of background subtraction used.

These factors cause variation in TL within and between gels (i.e. experiments, assays, TRFs). Using results collated for a number of species, we examined the sources of error and variability for the determination of mean TL.

4.1.1 Within and between gel effects

In general, the within-gel variation was found to be smaller than variation between gels. However, the magnitude of these effects can be different in each experiment if conditions are not carefully controlled.

Within-gel variation was largely determined by how well the gel had run (e.g. without distortion of the lanes) and could be fairly easily controlled with good technique in the laboratory. We found that based on the repetition of mollusc or crustacean samples three times per gel, the coefficient of variation for within-gel effects was one to three percent.

Between-gel variation is mainly driven by differences in signal intensity between lanes on separate gels. This was caused by differences in DNA loading levels between lanes and this can be compensated for by longer exposure times for lanes with lesser amounts of DNA. To control this source of error DNA concentration per sample and the amount of DNA loaded per lane required careful optimisation. This often involved repeating TRFs until the appropriate result was achieved. Coefficients of variation between gels (i.e. for replicate experiments) for Sydney rock oysters was 10-14% and this was typical across species.

4.1.2 Analysis of the effect of exposure time and background subtraction method

To estimate mean TL, an exposure time needs to be selected that is within the linear range of detection for the x-ray film. If exposure time is beyond these limits, the film will be saturated by the signal and it will not be possible to discern differences in the number of telomeres at each molecular weight.

In addition to this, at least three methods to control (i.e. subtract) for the effect of background signal on TL measurement are available. The background signal can be specific to the gel, the lane or the sizes of DNA fragments in the lane.

Three methods of background subtraction were evaluated in many of the TRF assays, including the exposure time experiment (Figure 2, Table 2). The three methods were:

1. **Minimum background subtraction**. In each lane the box with the lowest volume was subtracted from each of the remaining 24 boxes.
2. **Global background subtraction**. Two boxes were created on the autoradiograph in areas with no TRF signal (i.e. density). These were then averaged and the average quantity was subtracted from each of the boxes in the lanes. Therefore the background subtraction was constant for all boxes drawn on the autoradiograph.
3. **Adjacent background subtraction**. Background values were obtained by mathematically subtracting a linear background drawn between the box at the top of the lane (upper MW limit at which background occurs) and box at the bottom of the lane which best represents the lower molecular weight limit at which background occurs (lower background box) as per TELORUN for Excel v 1.4 (Harley *et al.* 1999).

An experiment was conducted to study the effect of exposure time and background subtraction method on mean TRF calculation. Exposures were made that transitioned between linear and saturation, with emphasis on exposures in the linear range. The effect on TL measurement was examined for six three-year-old oysters.

Exposure time had a significant effect on TRF determination (Figure 2), with a marked overestimation effect if exposure times were too short.

This highlighted the need to perform repeated exposures for each experiment to enable the correct exposure time to be selected. The best exposure will be the one in which signal from all DNA size classes are represented, but with no evidence of saturation. This can be examined by looking at the graph of the intensity of the signal down the lane using optical density measurement software (Godwin *et al.*, Submitted-a) (Appendix 5).

Table 2 shows the results for the comparison of background subtraction methods, which were fairly typical across species. The difference between the first two methods was minimal, whilst the third was more variable. The minimum background subtraction method (#1) is the method recommended in the Roche TeloTTAGG kit and is the most commonly used method in the literature. This method was adopted for assays in this project.

Table 2: A comparison of mean of telomere lengths (TL) values and coefficients of variation (CV%) for the three background subtraction methods for six three-year-old Sydney rock oysters (*S. glomerata*). C.V. is co-efficient of variation.

Subtraction method	Mean TL (n = 18)	Standard Deviation	CV%
1. Minimum	6499	1673	25
2. Global	6511	1481	22
3. Adjacent	9385	4346	46

4.1.3 Analysis of the effect of positioning 'boxes' and background subtraction method

Mean TL for each sample was estimated by scanning the exposed film and by measuring the optical density at each DNA length class. To facilitate these measurements, each sample lane was overlaid with a grid of 25 boxes.

Boxes could be copied exactly from lane to lane within a single gel but could not be exactly copied between gels. Although every effort was taken to maintain consistency, there was an element of subjectivity to the re-drawing and re-positioning of the boxes on subsequent images. Also, the lanes were not necessarily perfectly straight and therefore often required individual positioning of each box.

To examine the magnitude of the errors associated with this part of the analysis, repeated TRF measurements were made on the first eight lanes (i.e. eight school prawn samples) of a single gel. The process of drawing and positioning boxes and the molecular weight was conducted on four separate occasions and mean TL was calculated. Repeated TRF measurements on a single lane varied by between 121 and 420 bp, which represented 1.6-4.4% of the mean (Table 3). On average, CV for individual measurements of a particular sample was 1.1%.

Unlike the evaluation of background subtraction method and exposure time (above) where the 'Adjacent' method performed poorly, the background subtraction method had little effect on box variation in TL measurement. Average standard deviation for samples for background subtraction methods was 93, 95 and 120bp, which is equivalent to 1% CV.

The contribution of errors associated with drawing and positioning of boxes to overall estimation of mean TL was considered to be a minimal part of experimental error.

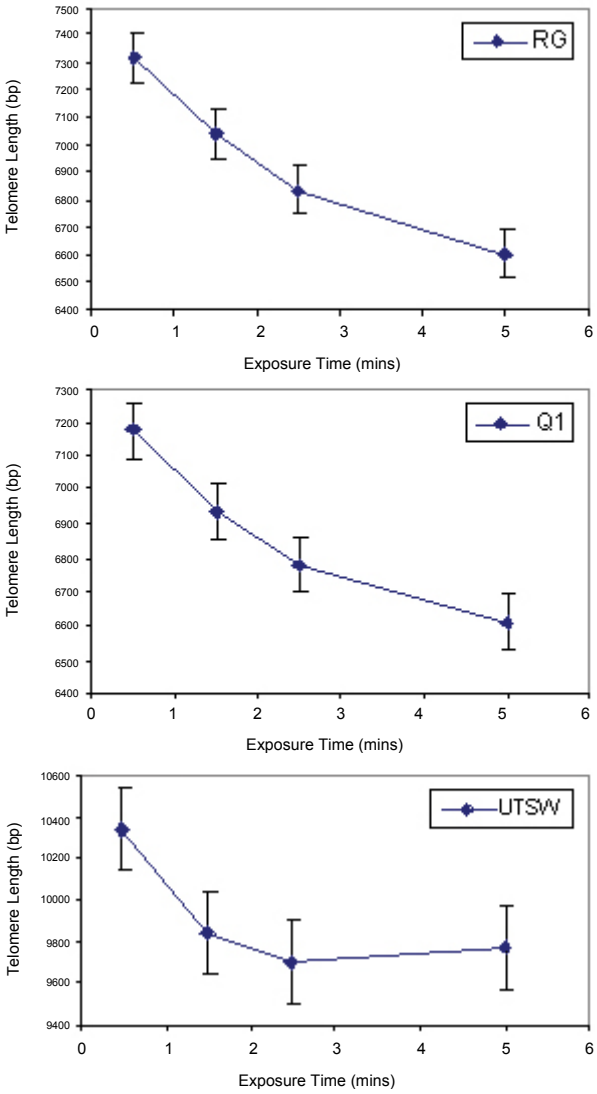

Figure 2: The effect of exposure time and background subtraction method (RG, minimum; Q1, global; UTSW, Adjacent) on mean telomere length estimates for six three-year-old Sydney rock oysters.

4.1.4 Further observations

In species where the telomere lengths are short (e.g. 3-5 kb), variability between repeated measurements and the difference between the methods of background subtraction were smaller than if telomeres were long (e.g. greater than 10 kb) or were close to the limit of resolution of the gel method (e.g. greater than 20 kb).

The effect of small experimental errors was magnified for longer telomeres because of the logarithmic way DNA migrates through the gel. Small physical differences higher up the gel cause large changes to TL measurements simply because increasingly large fragments run closer and closer together.

Table 3: A comparison of TL values for the three background subtraction methods, where boxes were positioned four times using eight school prawn telomere smears on a single gel.

	Prawn 1	Prawn 2	Prawn 3	Prawn 4	Prawn 5	Prawn 6	Prawn 7	Prawn 8	Overall
1. Minimum									
1st time	10532	7986	8518	9036	9051	7322	9264	7573	
2nd	10660	7962	8635	9200	9143	7498	9352	7695	
3rd	10746	8128	8655	9344	9292	7522	9523	7750	
4th	10737	8088	8639	9194	9192	7488	9386	7703	
Average	10669	8041	8612	9194	9169	7457	9382	7680	8776
SD	99	80	63	126	100	92	108	75	93
CV%	0.9	1	0.7	1.4	1.1	1.2	1.1	1	1.1
2. Global									
1st time	9985	7569	7485	8586	7896	6972	7857	6726	
2nd	10062	7576	7642	8806	8038	7154	7988	6869	
3rd	10111	7684	7630	8877	8102	7158	8098	6913	
4th	10236	7690	7568	8868	8088	7148	8093	6849	
Average	10098	7630	7581	8784	8031	7108	8009	6839	8010
SD	106	66	72	136	94	91	113	80	95
CV%	1.0	0.9	0.9	1.5	1.2	1.3	1.4	1.2	1.2
3. Adjacent									
1st time	10745	8521	8350	9075	9031	7638	9298	7588	
2nd	10906	8572	8688	9495	9225	7671	9401	7674	
3rd	11035	8697	8713	9476	9326	7744	9603	7710	
4th	10974	8683	8728	9444	9285	7608	9425	7690	
Average	10915	8618	8620	9372	9217	7665	9432	7665	8938
SD	125	86	180	199	131	59	127	54	120
CV%	1.1	1.0	2.1	2.1	1.4	0.8	1.3	0.7	1.3

4.1.5 Recommended standard method of analysis

The standard method of analysis adopted in the project consisted of:

- When comparisons were made of different tissue type, size or age cohorts, these were done on a single gel;
- Gels were replicated ideally at least three times;
- Samples were loaded in randomised order;
- One sample was repeated on the gel to provide estimate of the within gel effect (when appropriate);
- A number of exposures were taken to ensure the x-ray signal was stable and detection was optimal;
- Twenty-five boxes per lane were used for TL determination; and
- The background subtraction method used was the 'Minimum/lane' method.

4.1.6 Resources used to estimate telomere length per sample

As of September 2010, the cost to assay one sample for telomere length using the TRF method was $37.35. This consisted of $0.90 for the plastic tube used for storage of the tissue sample in liquid nitrogen, $4.26 for the extraction of genomic DNA from that sample, and $31.68 for restriction enzyme digestion, electrophoresis, transfer, hybridisation, chemiluminescence and miscellaneous costs (Table 4). The time taken for one person to assay 36 samples (i.e. two experiments at once) was eleven days for the work in the laboratory, plus one to two days for TL measurement and statistical analyses (Table 4).

Table 4: Estimates of the costs and time taken for the five main procedural steps for terminal restriction fragment (TRF) analysis.

Procedural Step / Item	Time	Approx. cost per sample ($)
1. Sampling and storage of fresh tissues		
Collection of animals	One day	Variable
Freight	Generally paid by sample collector	N/A
Dissection	One day for 30 samples using two people, depending on how many tissues are taken and how difficult it is to dissect the animals	N/A
Storage cryo tubes	N/A	0.90
Storage in liquid N dewars	15 L per month over three years = 540 L. Cost per 30 litres $33.24.	Total cost to maintain storage in dewars was at least $600 over 3 years.
2. Extraction of total genomic DNA from these tissues.		
	Two days are required to extract and check the quality of the DNA from one to 12 samples.	4.26
3. Terminal Restriction Fragment (TRF) assay		
a. Digestion and electrophoresis	1-2 days	4.70
b. Transfer	1 day	1.74
c. Hybridisation and chemilumin-escence	1 day	1.76
d. Miscellaneous costs		1.4
	Total was four days for a maximum of 36 samples	Total was 10.56
4. Calculation of Mean TL for each sample from resulting autoradiographs.		
	½ -1 day to process one blot (using 3 film exposures/blot).	N/A
5. Statistical Analyses		
	½ -1 day to analyse data from one species.	N/A

4.1.7 Real-time PCR as an alternative method to TRF assay

The project developed a standardised method of estimating telomere length using TRF assay, where the CV between experiments was 10-14%. While this compares favourably to the literature, the method is time-consuming (13 days for 36 samples for one FTE) and expensive ($37.35 per sample without labour).

Quantitative PCR (Q-PCR) is an alternative (Callaghan *et al.* 2008; Cawthon, 2002), but protocols for non-model species have not been published. Major issues include finding reference genes and designing primers for the target species. Q-PCR is expected to be significantly faster (e.g. two days for 36 samples for one FTE) and less expensive (e.g. $10 per sample). However, the accuracy and precision of the Q-PCR method requires careful evaluation (Dunshea *et al.* 2011).

4.2 Molluscs

4.2.1 Sydney rock oyster (*Saccostrea glomerata*)

The Sydney rock oyster is a valuable cultured species in Australia and occurs in inter-tidal areas from the New South Wales and Victoria border area (37°S) to around Townsville (19°S) in Queensland, Australia (Nell, 2001; O'Connor and Dove, 2009). The species' ready availability at known, absolute ages from oyster farms at different latitudes made it an ideal candidate upon which to develop techniques and test the hypothesis that telomere length differs with age.

Telomere length declined with age in Sydney rock oysters. But, the levels and attrition rates were variable, differing by region, tissue type and year of sampling. Telomeres appeared to shorten more rapidly in Moreton Bay than they did in Wallis Lakes, though the young oysters in Moreton Bay started with longer telomeres. The telomere lengths of three- and four-year-old animals from both locations were similar (7,500-8,500 bp) (Appendix 5.1, Godwin *et al.*, Submitted-a).

Although telomere attrition with age in oysters conformed to our expectations, the variability with region and time of sampling remains to be investigated further, but could be associated with other cellular processes involved in the aging process.

In Moreton Bay, telomere lengths for mantle tissue were shorter than gill tissue, whereas the relationship was reversed for Wallis Lake oysters. Gill tissue turnover rate is apparently higher than mantle in molluscs, so the expectation is that gill telomere lengths would be shorter, or perhaps decrease at a faster rate, than in mantle.

Despite the relationship being influenced by extrinsic and intrinsic factors, it is likely that the telomere length could be used to sort oysters into two classes; 'young', compared to 'old', for example. While this is not as satisfactory as being able to age samples into tighter age-classes, there are some species where this information would be useful to fisheries management (e.g. spanner crabs).

4.2.2 Tropical abalone (*Haliotis asinina*)

The oyster TRF method transferred to abalone with minimal modification or optimisation. TRF assays were conducted on muscle tissue from seven individuals from each age class.

Results showed that telomere length did not differ significantly across the measured age range, although there was a trend for telomere length to be longer in the 18 month age class (Table 5). This was unexpected since we observed a decrease in oysters and theoretically telomere length should decrease with age. The results may be explained because of the small number sampled in each age cohort or that there was only a seven month age difference between the youngest and the oldest tropical abalone sampled. This difference represents only a fraction of the abalone life-span, which is thought to be approximately fifteen years.

Tropical abalone samples were used to estimate intra and inter-gel variation. A single sample was assayed (abalone Haas 7) three times on two different experiments (Gel 1, Gel 2; Table 6). Gel 2 was run as a training exercise, and consequently the coefficient of variation in Gel 2 (25%) was greater than in Gel 1 (1.8%). Gel 2 was not seated in the tray correctly, resulting in uneven migration of telomere DNA smears. The transfer to nylon membrane was also affected when the Gel 2 was fractured during the transfer. Results such as this were excluded from datasets.

Table 5: Mean telomere length (bp) for tropical abalone (*H. asinina*) aged 11 and 18 months.

Age class	n	Mean Telomere Length (bp)	SD
11 months old	7	4831	592
18 months old	7	5482	502

Table 6: Repeatability of average, standard deviation (SD) and the coefficient of variation (%CV) of telomere length (bp) for the same tropical abalone sample (abalone Haas 7), run three times on two replicate gels (see text for explanation of differences between gels).

	Gel 1	Gel 2
Average	5044	4805
SD	93	1195
%CV	1.8	24.8

4.2.3 Black lip abalone (*Haliotis rubra*)

TRF assays on temperate abalone were conducted on cultured, hybrid (*H. rubra* x *H. laevigiata*) individuals spanning an age range of 2-4 years. No significant difference in telomere length was found between the age classes of the hybrids.

The telomere lengths of older, wild-caught black lip abalone (*H. rubra*) were estimated from large individuals from George III Rock Research Area, southeast Tasmania from a wild, fisheries population. Shell sizes measured 140-153 mm and the abalone were estimated to be 7-10 years old based on the size and condition of their shells (S. Frusher, pers. comm.). Genomic DNA was extracted and three replicate TRF assays were conducted on ten individuals. Estimates were confirmed later by repeating this procedure.

Telomere length analysis was also performed on smaller, wild-caught black lip *(H. rubra)* abalone from George III Rock. Shell size classes were 30-40 mm (n = 4), 41-50 mm (n = 8), 51-60 mm (n = 6), 61-70mm (n = 7), 71-80 mm (n = 2) and 80-90 mm (n = 3).

There was a relationship between abalone shell size and mean telomere length, with a highly significant negative correlation (Figure 3). Telomere length for small black lip abalone (*H. rubra*, shell size 40-90mm) ranged 2.7-4.3 kb and were approximately twice the length of telomeres in large abalone (shell size 140-153 mm, 1.5-2.1 kb). Hybrid abalone (*H. rubra* x *H. laevigiata*) telomere lengths appear to match the smaller, wild-caught abalone (Figure 3) (Appendix 5.2, Godwin *et al.*, in prep.).

If this relationship was able to be repeated spatially and temporally, and shell size converted to age, this result suggests that it may be possible to develop telomere length as a robust biomarker for age in temperate abalone (*Haliotis spp.*).

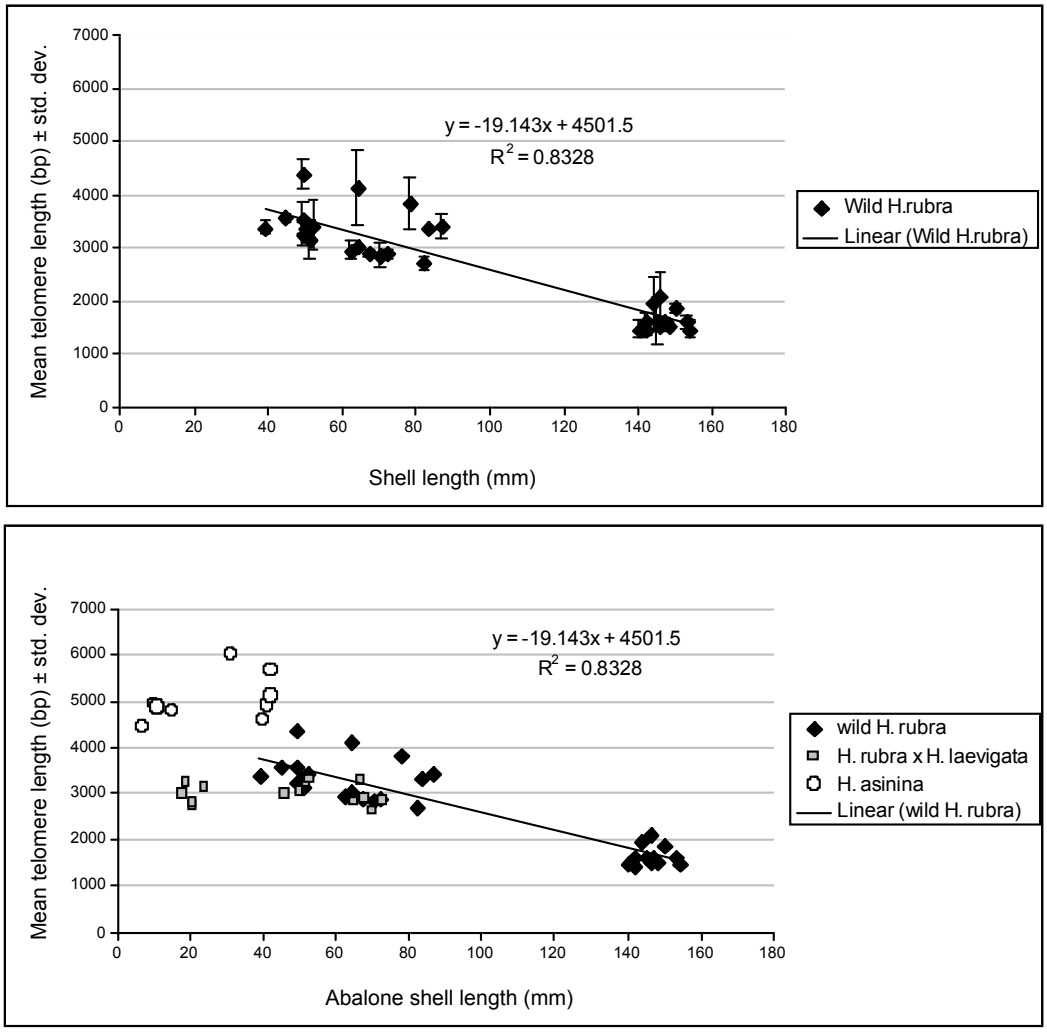

Figure 3: The relationship between telomere length (bp) and shell size (mm) for wild caught *Haliotis rubra* (♦) with corresponding linear regression (upper panel) and *H. asinina* (○) and *H. rubra* x *H. laevigata* hybrids (■) compared to (♦) *H. rubra* (lower panel).

For abalone with similar shell sizes, telomere length appears to vary. For example, amongst abalone with shell sizes of 40-80 mm, five abalone had TL above the average for that shell size. There is a growing literature that links telomere length to condition, such as stress (e.g. Salomons *et al.* 2009), where short telomeres may be indicators for poor health and premature death implying that animals with longer telomeres may be more resistant to stress and thus have higher growth rates and survivorship. Abalone have variable growth in the wild, but the causes are unclear. Further research is suggested to test whether TL could estimate the percentage of animals in a population that were affected by stress and categorise populations as healthy or stressed. Size limits for harvest may differ between stressed and normal populations.

4.3 Crustaceans

4.3.1 School prawn (*Metapenaeus macleayi*)

Our experiments showed that school prawn telomeric DNA was highly susceptible to degradation, which had a major impact on the results for this species. Degradation was presumed to occur during 4°C storage in the laboratory (after it had been extracted from the tissue source) and prior to (and during) TRF assays, but it could also occur at other times (e.g. during tissue storage and collection).

The effect of degradation was most pronounced for experiments on large prawns, where up to 24 days elapsed between the extraction of DNA from tissue and TRF assays. For example, experiments 1, 2 and 3 were conducted nine, 16 and 24 days post extraction for large prawns (Table 7). Statistical analyses showed telomere length values decreased significantly as the number of days that elapsed between DNA extraction and telomere length assay increased (Figure 4).

Table 7: Mean TL measurements (bp) for large school prawns showing consistent decrease in TL with subsequent replicate experiments. Some measurements were missing (*).

Sex	Sample	Experiment 1	Experiment 2	Experiment 3	Average
Female	102	7141	6406	4710	6086
	103	6262	7525	6720	6836
	104	7594	7960	5432	6995
	105	6911	6959	*	6935
	106	6624	8863	5454	6980
	107	11862	10312	7512	9895
	108	10372	8760	8052	9061
	109	6553	7229	5879	6553
	110	10350	9650	6517	8839
	111	13645	10003	8255	10634
	112	10986	11785	8789	10520
Male	126	7459	7654	5615	6909
	127	7755	7744	6560	7353
	128	8769	8779	6749	8099
	129	12268	8303	5253	8608
	132	8618	8223	6649	7830
	134	12636	10864	9080	10860
	136	12091	10901	7542	10178
	137	9598	9944	8193	9245

This means that if genomic is DNA is stored for a number of weeks before assays are conducted, observed telomere lengths could be around thirty percent lower than they might have been if the assays had been conducted with five days of extraction.

This degradation effect occurred despite quality control measures. Before each TRF assay, extracted DNA was assessed for degradation by running into an agarose gel. Following convention, DNA that appeared as a single high molecular weight band was assumed to be undegraded (Figure 5) and was used in TRF assays. A second quality control measure was also implemented; TRF assay results per individual were removed from the analysis if they showed (1) presence of an elongated telomere smear instead of a discrete smear, and (2) absence of sub-banding pattern (Figure 6).

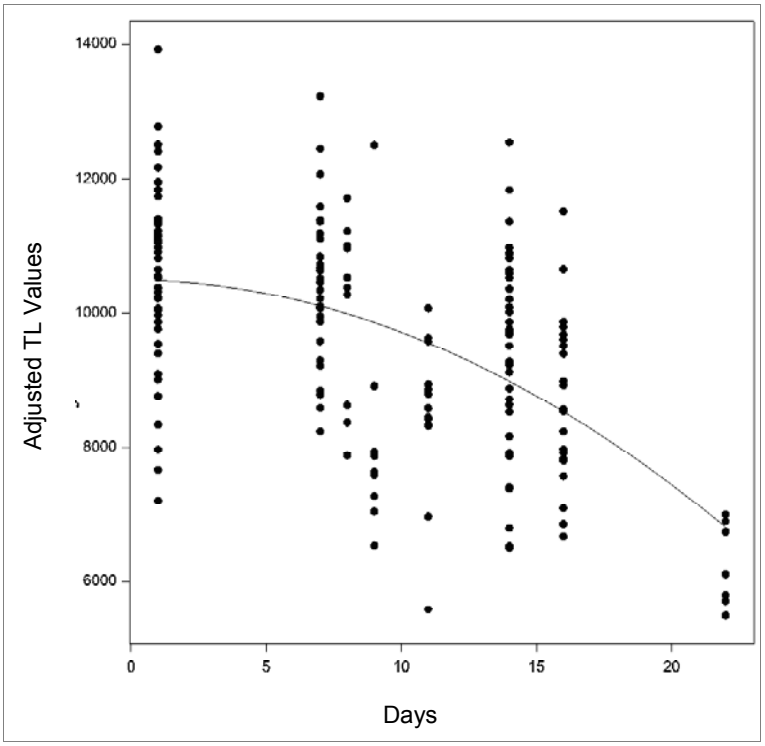

Figure 4: The effect of storage time measured in days that elapsed between DNA extraction and telomere length assay on telomere length values (bp) based on observations from prawns across three replicate experiments.

Despite these quality control measures, telomere length was observed to decline with time since DNA extraction (Figure 4). This implied that the observation of a high molecular weight band on an agarose gel was not a reliable test for undegraded (i.e. intact) telomeres and the second quality control measure (i.e. removal of TRF results) may not have been sufficiently stringent to control for degradation.

The observed result would be consistent with the degradation (i.e. presumably oxidation) of the telomeric ends of the chromosomes only, leaving the majority of the genomic DNA intact. Presumed oxidation of school prawn telomeric DNA occurred despite the addition of the reducing agent DTT to the DNA storage buffer.

The degradation effect may be unique to the telomeres of school prawns. We know that telomeres are subject to degradation via oxidation (E. Blackburn, pers. comm.) and based on our results, we know that school prawn telomeres may be particularly susceptible. Further research is needed in this area, as oxidation would also potentially bias telomere lengths in this species assayed by alternate techniques, such as quantitative PCR (Section 6, Further Development).

Compared to the genomic DNA of spanner crabs (see Section 4.3.4), about five to ten times more school prawn DNA was needed per TRF assay per sample to give an observable result on x-ray film. This may suggest that school prawns have larger and less numerous chromosomes (i.e. fewer telomeres) than crabs. This may be a reason to develop quantitative PCR method to measure telomere length in school prawns since this method does not rely on using large quantities of intact genomic DNA. However, telomere oxidation would need to be accounted for.

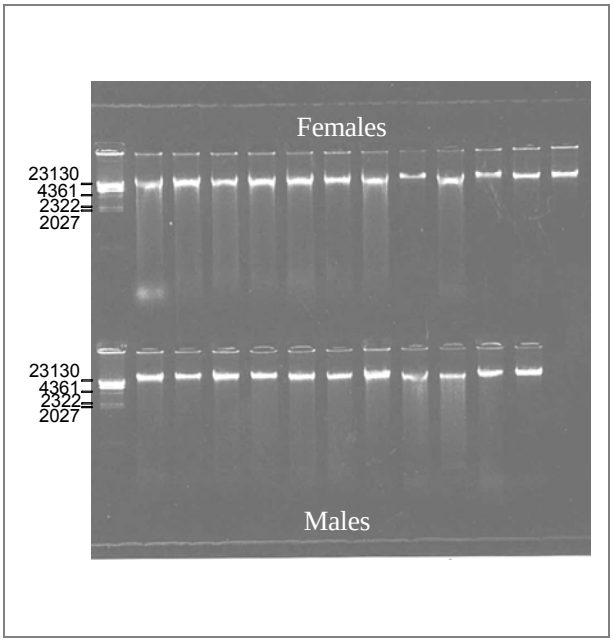

Figure 5: Examples of extracted DNA that was judged to be undegraded before TRF assay but where telomeric DNA was later shown to be affected by oxidative damage. DNA was extracted from 12 female (upper panel) and 11 male medium sized school prawns. Molecular weight markers (bp) are shown on the left hand side.

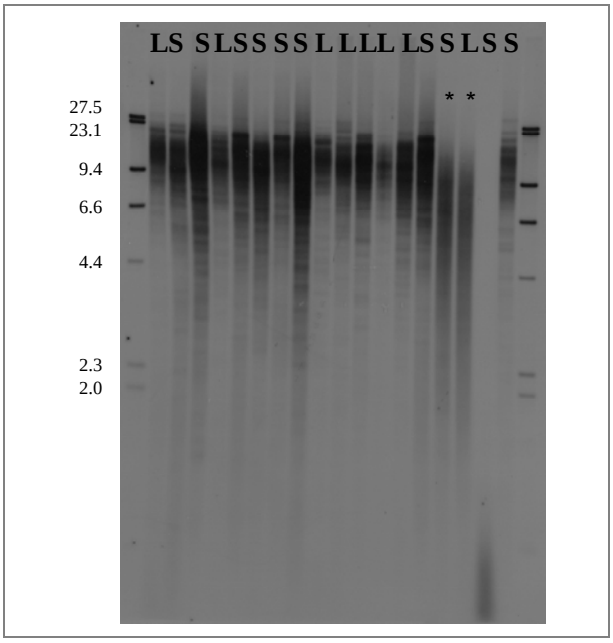

Figure 6: TRF assays from small (S) and large (L) school prawns showing contrast between results on undegraded and degraded DNA. Two lanes containing degraded DNA are marked with asterisks. Size of molecular weight markers (kb) are shown.

After taking account of degradation of school prawn telomeres during storage in the laboratory or during the TRF assay, there was no significant difference in telomere length for males or females of varying carapace lengths (Figure 7) (Appendix 5.3, Godwin *et al.*, Submitted-b)

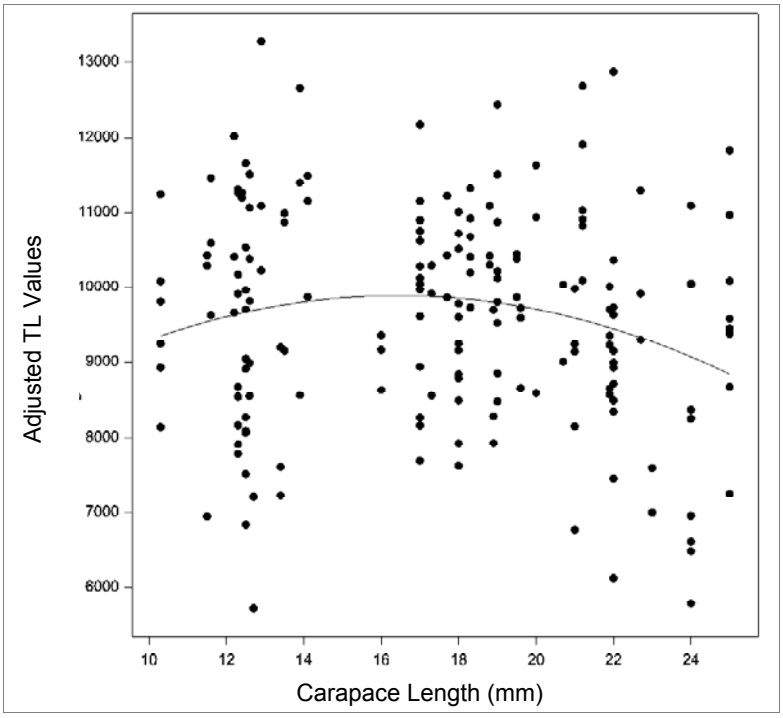

Figure 7: The effect of size, as indicated by carapace length, on telomere length values of 29 male and 34 female school prawns, *Metapenaeus macleayi*, based on estimates from three replicate experiments.

4.3.2 Eastern rock lobster (*Sagmariasus verreauxi*)

Assays to estimate telomere lengths of *S. verreauxi* were conducted on muscle and gill DNA using (i) six pueruli (carapace length 11-15 mm), (ii) five samples of three-year-old individuals (carapace length 65-87 mm), (iii) six large (carapace length 103-130 mm), and (iv) five very large (carapace length 150-159 mm) individuals.

Telomere lengths means were close to 16-18 kb; approximately twice that which was recorded for school prawns. There was little variation in telomere lengths between the sampled tissues of *S. verreauxi* (Table 8). Amongst adult lobsters there was no difference in telomere lengths between gill and muscle tissue ($F_{1,94}$ = 2.89; P = 0.092). The mean telomere length of puerulus tail tissue was 17,908 ± SE 366 bp, which was also not significantly different from the combined mean of 16,961 ± SE 1,131 bp for gill and muscle tissue of older lobsters (t = 0.80; P = 0.43).

No relationship between age and telomere length was detected amongst the *S. verreauxi* in this study. The effect of age (based on size class) on telomere length was only tested amongst adult lobsters. Data obtained from pueruli were excluded since they were based on a mixture of tissues, whereas telomere lengths of older lobsters were based on single tissues (either gill or muscle tissue). Amongst the remaining three size classes (three years old, large and very large lobsters) there was no significant difference in telomere length (Table 8). Furthermore, when carapace length was used as a more precise measure of size and the relationship between telomere length and carapace length tested by linear regression the result was also non-significant. The coefficient of variation (CV) amongst pueruli was 11.6% while amongst adults the CV was 21.2%.

Like school prawns, the number of days that elapsed between extraction of *S. verreauxi* DNA and the corresponding TRF assay had a significant effect on telomere length estimates. Some *S. verreauxi* DNA extracts had been stored for nearly ten months before assay and there was an average decline in telomere length estimate of 24.8% over 300 days. The degradation observed for *S. verreauxi* occurred more slowly than that observed for school prawns, where a similar degree of degradation occurred in a tenth of the time (Appendix 5.3, Godwin *et al.*, Submitted-b).

Table 8: Mean and standard errors (SE) for telomere length estimates from different tissues (a) and size classes (b) of eastern rock lobster, *Sagmariasus verreauxi*.

(a) Tissue	Telomere length (bp) Mean ± SE
Puerulus tail (Abdomen)	17908 ± 366
Adult, gill	17603 ± 565
Adult, muscle	16318 ± 486
Adult, gill muscle combined	16961 ± 1131

(b) Size class	Telomere length (bp) Mean ± SE
Small (3 years old)	16720 ± 618
Large	18190 ± 785
Very Large	15961 ± 630

4.3.3 Southern rock lobster (*Jasus edwardsii*)

Telomere length assays were successfully conducted on *J. edwardsii* individuals using DNA extracted from tail muscle tissue of nine pueruli and muscle tissue of nine adults (Figure 8). Gill tissue was unavailable for assaying. Two of the puerulus samples degraded during the assay and therefore results are presented for the seven pueruli and nine adults.

Average telomere lengths for pueruli were 11,205 bp, which was significantly shorter than for adult *J.edwardsii* with 15,200 bp (Table 9). These telomere lengths are intermediate between the average telomere lengths recorded for school prawns and *S. verreauxi*. The occurrence of shorter telomeres in puerulus, compared to adults, was counter to our expectations. Further experiments are recommended given this and the magnitude of the difference, which was about twenty percent.

In adult *J. edwardsii* the relationship between carapace length and telomere length was non-significant ($F_{1,7}$ = 0.37; P = 0.563). Carapace lengths of the nine adults assayed ranged 80-120 mm (Appendix 5.3, Godwin *et al.*, Submitted-b).

The number of days that elapsed between DNA extraction and TRF assay did not significantly affect telomere length estimates of *J. edwardsii.* However, susceptibility of *J. edwardsii* DNA to degradation cannot be ruled out, because the difference between the shortest and longest amount of time that elapsed between DNA extraction and TRF assay was only ten days (18 days vs. 28 days).

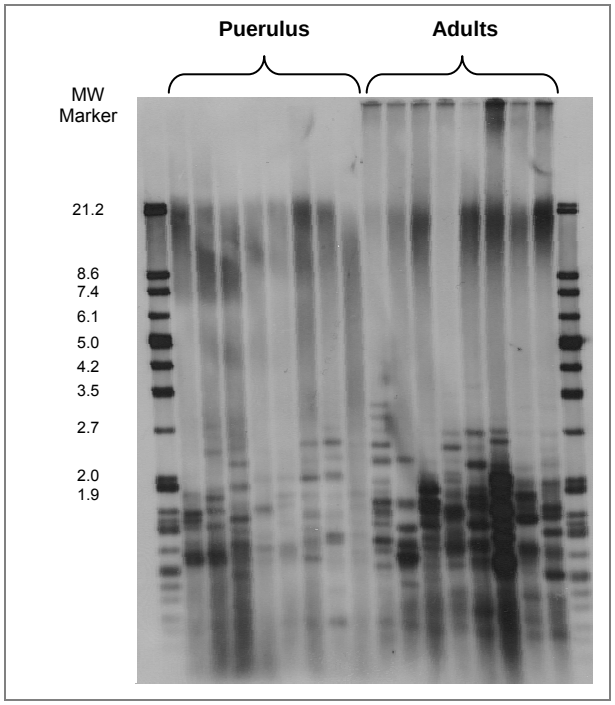

Figure 8: Autoradiograph of fluorescently-labeled telomeric restriction fragments (TRF) of young (puerulus) and older (approximately eight years old) southern rock lobster (*Jasus edwardsi*). DNA size standards are in the outer lanes.

4.3.4 Spanner crab (*Ranina ranina*)

Telomeres of spanner crabs were difficult to assay in the laboratory. The problems we encountered were due to such issues as:

1. 'Well hang-up', where digested DNA did not move as expected into the gel;
2. Incomplete digestion of genomic DNA;
3. Non-specific hybridisation of the telomere probe to DNA throughout the gel; and
4. Apparently long telomeres, which were beyond the limit of separation by conventional electrophoresis.

We took an experimental approach to solving these issues, and some progress was made. However, the accuracy of the measurements produced was compromised, and further research is recommended.

Our experimental approach included:

1. A more rigourous DNA extraction method was used, including a salting-out step (Miller *et al.* 1988) and a phenol chloroform step. This method improved 'well hang-up', but did not completely eliminate it. The improvement was attributed to the exclusion of cellular components (e.g. chitin, polysaccharides) that co-purified with genomic DNA. Exclusion of these products may have also improved the efficiency of enzyme digestion on genomic DNA.
2. Different restriction enzyme combinations were tested compared to the standard combination.

Table 9: Telomere length estimates (bp) for sixteen *J. edwardsii* individuals obtained from three replicate experiments. The mean and standard deviation for individual estimates is presented, as well as the average and standard deviation amongst individuals for each size class. Some values for pueruli were missing (*).

Sample ID	Carapace length (mm)	Rep 1	Rep 2	Rep 3	Individual Average	SD between reps
Adults						
261108	80	14058	16111	15711	15294	1088
261113	112	16894	17867	18752	17838	929
310408	119	14994	15953	16182	15710	630
310420	119	16133	15706	17466	16435	918
310510	119	15122	15272	15962	15452	448
310535	111	16103	15717	16133	15984	232
310540	108	17988	16404	15084	16492	1454
310543	120	10051	9222	11663	10312	1241
310550	118	14422	12651	12781	13285	987
			Adult Average		15200	881
Pueruli						
P1	unknown	13224	13914	13061	13400	453
P2		*	13480	12764	13122	507
P4		12171	12634	11669	12158	482
P5		9609	7541	7062	8071	1354
P6		8743	7894	7407	8015	676
P7		11208	10917	11787	11304	443
P12		12365	*	*	12365	*
			Pueruli Average		11205	653

3. Digested DNA was separated using FIGE (Fluctuating inverted field electrophoresis) using a method designed to separate large DNA (25-150 kb) fragments.
4. Hybridisation and washes were performed at higher temperatures to improve stringency, and hence reduce non-specific hybridisation.

As part of these experiments, two TRF assays were performed using DNA isolated from gill tissue, and eight small individuals and eight large individuals were tested. Gel A DNA was digested with *Rsa*I and *Hin*fI and gel B DNA digested with *Alu*I and *Hpy*Ch4V.

The TRF assays were cleaner with much less non-specific hybridisation than previous attempts. The DNA was well separated by the FIGE method and the *Alu*I/*Hpy*CH4V enzyme combination resulted in more efficient digestion than *Rsa*I/*Hin*fI. However, in this experiment another problem was encountered; DNA degradation appeared to occur during the experiment, which was more noticeable amongst the small individuals.

Telomere lengths (TL) from this experiment are presented in Table 10. The range in TL recorded for both small and large individuals was approximately 8-25 kb. Apart from sample Rara20, telomere lengths were quite similar between the two gels for each individual. The low values recorded for samples Rara88, 14 and 35 compared to the others are probably a result of partial DNA degradation. In general, the results for *Alu*I/*Hpy*CH4V digested DNA were lower than the results obtained for *Rsa*I/*Hinf*I digestion by about 2 kb. This suggests that *Alu*I/*Hpy*CH4V enzyme cleavage sites are closer to the true telomere repeats than *Rsa*I/*Hinf*I sites (Appendix 5.3, Godwin *et al.*, Submitted-b).

Table 10: Mean telomere length (bp) from gill tissue for small (carapace width less than 85 mm) and large (carapace width greater than 115 mm) spanner crabs (*Ranina ranina*) where genomic DNA was digested with two sets of restriction enzymes (Gel A, enzymes *Rsa*I and *Hin*fI; Gel B, enzymes *Alu*I and *Hpy*CH4V). Some samples degraded completely during the procedure (NA) and others were partially degraded (*).

Sample ID	Size Class	Gel A	Gel B
Rara01	S	13583	11369
Rara05	S	NA	NA
Rara16	S	NA	NA
Rara21	S	NA	NA
Rara24	S	NA	NA
Rara87	S	22 096	25 177
Rara88	S	3 020*	NA
Rara89	S	NA	NA
Rara06	L	8 082	8095
Rara14	L	2718*	NA
Rara20	L	14 836	23 426
Rara35	L	4 825*	NA
Rara68	L	20 283	23 117
Rara69	L	15 601	12 768
Rara120	L	14 838	10 211
Rara121	L	9 982	NA

Further research would be required to further optimise experimental conditions before reliable estimates of telomere length can be made in this species, such as:

1. Removing contaminants (e.g. chitin) from genomic DNA extractions, which may be affecting the mobility of the DNA in electrophoresis (i.e. 'well hang-up');
2. Testing alternative tissue sources, such as blood (i.e. free of chitin);
3. Using the FIGE electrophoresis system as it would be likely to give results with the large spanner crab telomeres. For instance, mouse telomeric DNA up to 150 kb was successfully resolved by a similar system (i.e. pulsed field electrophoresis; M. Brown, pers comm.); and
4. Using a DIG-labelled molecular weight size standard that encompasses the upper range of telomere lengths (e.g. greater than 20 kb).

4.4 Vertebrate control

4.4.1 Silver perch (*Bidyanus bidyanus*)

Silver perch was included in this project as a representative vertebrate species to ensure that the TRF method used here on invertebrate species would also obtain the predicted results from vertebrates, which have been more widely studied in the literature. This part of the project was used to train an Honours student (Ms. Kendy Chuang).

Silver perch samples were obtained from a government aquaculture farm in northern New South Wales. Three tissues (heart, liver and skeletal muscle) were obtained from one-, two- and three-year-old fish. Three-month-old fish were also sampled, but because of their small size, individual tissue types could not be obtained. Telomere lengths were measured with the standard TRF method. Silver perch telomere smears are shown in Figure 9.

Silver perch telomeres were relatively short, between 3,000 and 5,000 bp. Telomeres were longest in muscle (4,249 bp) and shortest in heart (mean 3,531 bp). Liver telomeres were intermediate in length (3,769 bp). Tissues can differ in their telomere length because of the different cell turnover rates in each tissue, but telomere length can also vary between chromosomes. Muscle is less mitotically active than liver and heart, which may account for our observation of longer telomeres.

Telomere length in three-year-old fish was shorter than two-year olds, as expected. However, telomere length in one-year-old fish was shorter than two-year-old fish, which implies an increase in telomere length between one and two years, followed by a decrease from two to three years. This could be a sampling effect, as the differences between the age-classes was slight (although significant) and only young age classes were sampled from the life-span of the species, which is thought to reach ages exceeding ten years in nature. This project did not assay for the enzyme telomerase that counteracts telomere attrition. It is possible that the expression of the enzyme varied between age-classes that affected the expected relationship between age and telomere length (Appendix 5.4, Chuang *et al.*, in prep.)

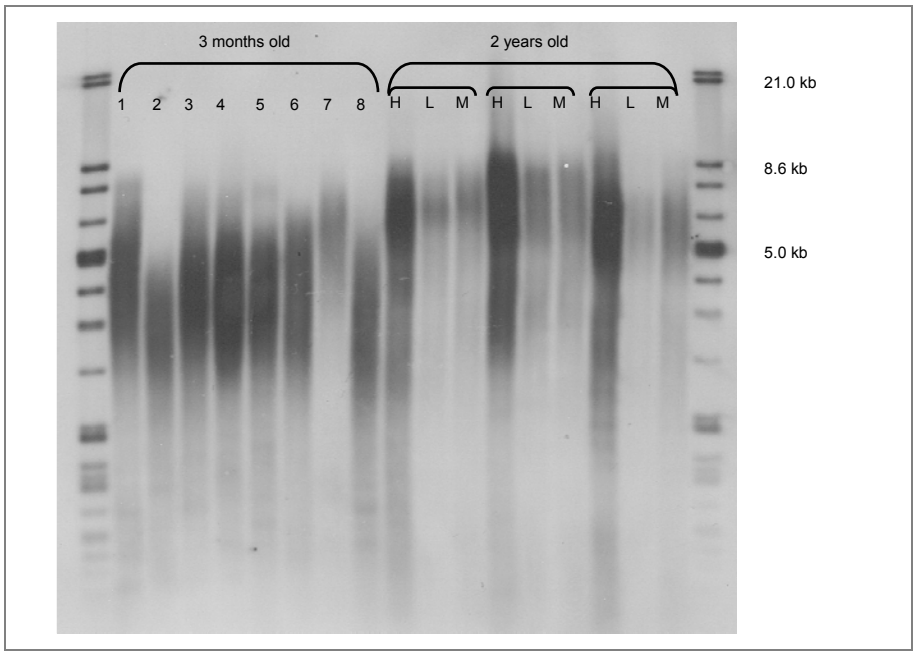

Figure 9: Autoradiograph showing telomere smears following TRF analysis of silver perch DNA extracted from whole bodies of eight three-month-old individuals and heart (H), liver (L) and muscle (M) tissue from three individuals two years of age.

5. Benefits and Adoption

The outcomes of this work will benefit the commercial fishing industry, particularly the sector that is reliant on invertebrate species. It will also benefit researchers and managers working on these resources, as well as the general scientific community who are interested in the age-structure of naturally occurring species, which are difficult to work on using conventional age-determination methods.

6. Further Development

6.1 Procedural recommendations

New methods are needed to assay telomere length in the laboratory, as the method used in this project (TRF assay) was time-consuming and expensive. Other methods are available to estimate telomere length, such as flow cytometry (Baerlocher *et al.* 2003), fluorescence *in situ* hybridisation (Guo and Allen, 1997) and quantitative PCR (Callaghan *et al.* 2008; Cawthon, 2002). Quantitative PCR appears to have the most promise – it has the potential for high-throughput, can be performed on tissue taken non-lethally and is estimated to be less expensive (~$10 per individual) than the TRF method. If a Q-PCR method could be developed for fisheries species (e.g. abalone) it would be ideal for surveying population samples. This study found that telomeres of crustaceans degraded following storage of extracted DNA in the laboratory at 4°C and during experimental manipulations, which could potentially bias Q-PCR results for these species. Dunshea and others (2011) suggest that issues such as PCR efficiency and non-terminal telomeric DNA (i.e. telomeric DNA located within, not at the ends of chromosomes) need to be considered in assay design.

Tissue collection and storage methods that facilitate field and laboratory analyses are important for the application of any method of telomeric DNA analysis across large numbers of samples per species. Non-lethal sources of tissue need to be investigated across taxa. Blood can be obtained non-lethally from most organisms and skeletal muscle biopsies can be taken from fish and crustaceans. Blood can contain nucleases (i.e. enzymes that degrade DNA) and DNA extractions from blood were often performed from fractions that were enriched for blood cells only. Unlike avian DNA, blood from fish and invertebrates contains low numbers of nucleated cells, reducing the amount of DNA that can be extracted from that tissue.

This project attempted a ‘blind’ experiment, where the relationship between telomere length and age in Sydney rock oysters was used to estimate the age of ‘blind’ (i.e. where age was unknown to the investigator) samples based on their telomere lengths. A colleague from the University of Queensland kindly assisted in this trial. While this type of experiment appears to be a tempting way to convince others of the usefulness of a DNA biomarker for age, the implication is that some factors affecting the relationship were not fully explored in the experimental framework. A well-designed study will encompass all possible variables, and accuracy, precision and reliability will be assessed by comprehensive statistical analyses. ‘Blind’ experiments work well in some fields, for example consumer reactions to seafood products, but not in the context of a study such as this.

Further development is needed to establish telomeric DNA as a biomarker for age in species where known age individuals cannot be obtained and where the need for reliable age information is high. Without age information, it is not possible to calibrate telomere-length rate of change per time unit. This may include long-lived species such as the Queensland groper (*Epinephelus lanceolatus*), giant clams (*Tridacna gigas*) and species that cannot be practically grown in captivity (spanner crabs, *Ranina ranina*). This could be dealt with in some cases by taking tissue samples (non-lethally) from the same animal across its lifetime and assaying for telomere length. However, this would require reliable recapture of the animal through time. This may be possible as part of a mark-release-recapture program, perhaps facilitated by some sort of restraint to dispersal in the wild to assist recapture. The latter may be relevant to spanner crabs, not needed for giant clams but impossible for species such as groper.

6.2 Species-specific considerations

Methodological issues aside, we observed in this project that telomere length was probably not a useful biomarker for crustaceans. Spanner crabs had the longest telomeres (possibly up to 25,000 bp), which were difficult to resolve using the TRF assay and we suspected that spanner crab DNA had other characteristics (e.g. contaminants) that made it difficult to work with. In other crustacean species, telomere length was not proportional to body size in school prawns, eastern rock lobster (*S. verreauxi*) and most likely southern rock lobster (*J. edwardsii*). Telomere length estimates for the smaller size class (i.e. puerulus) for *J. edwardsii* were poorly resolved. Given the urgent need for age information in fisheries for these species, further research on the apparent absence of correlation between telomere length and age in crustaceans is important. Attention should be given to the susceptibility of crustacean telomeric DNA to degradation during storage in the laboratory and the possibility that the enzyme telomerease is up-regulated in crustacean tissue.

One explanation for the lack of correlation in crustaceans could be the role that the enzyme telomerase takes in crustacean tissue. Telomerase can be thought of as a 'brick-layer' that replaces telomeric DNA repeat units that are lost at each cell division, although is likely that its role is more substantial than this simple model (Blackburn, 2009; Blackburn *et al.* 2006). Klapper and others (1998) detected high telomerase activity in several tissue types in the lobster *Homarus americanus*, which they interpreted as a mechanism to preserve chromosomal function during the slow onset of senescence in this species. Persistent telomerase expression has also been detected in a wide range of adult somatic tissues of the prawn (*Penaeus japonicas*), green crab (*Carcinus maenus*) and a range of other marine invertebrates. It has been suggested that this is necessary in organisms with high regenerative ability (Elmore *et al.* 2008; Lang *et al.* 2004). An assay for telomerase is commercially available (i.e. TRAP assay), which could be applied across tissue types of crustacean species from this study. If telomerase activity is elevated in long-lived species, activity should be high in *J. edwardsi* and *S. verreauxi* that have an estimated lifespan of thirty to fifty years, but lower in the short-lived school prawn.

6.3 A biomarker for age or body condition?

In addition to the use of telomere DNA as a biomarker for age, there is also the possibility of using it as a biomarker for condition. Monaghan (2010) evaluated a decade of research on telomeric DNA in an ecological setting. As well as reviewing the general decline of telomere length with age, Monaghan (2010) evaluated the link between telomere length and body condition. Monaghan proposed that stress, possibly oxidative stress, derived from the environment affects telomere length among individuals, which causes widespread damage in addition to shortening telomeres. Individuals having shorter than average telomeres may be more susceptible to premature death, and Salomons and others (2009) have shown that individual birds whose telomeres shorten faster than normal have lower chances of survival. This suggests that assays of the inter-individual variation in telomere length, as well as assays to determine the rate of decline of telomere length, may be a valuable pathway to developing a biomarker for body condition and to understand the role of telomeres in the evolution of life histories.

Authorities that manage Australian resources of marine molluscs, such as abalone and scallop, require population estimates on age and body condition. The Tasmanian abalone (*Haliotis rubra*) supports the largest wild abalone fishery in the world, supplying more than a quarter of global catch. Several lines of evidence suggest that body condition varies between populations. Genetic connectivity has been shown to be low and sporadic between populations (Miller *et al.* 2009), depletion of some population has occurred despite active management, growth rates are variable between populations and mortality has been up to 90% in populations have been affected by viral ganglioneuritis (AVG). A biomarker of body condition could give early warning of viral infection as well as lead to a revision of size limits on harvest if stunted-growth, small-shelled populations where shown to be in poor condition rather than younger than same-sized conspecifics.

Likewise, there is an imperative to understand body condition parameters in commercial scallop (e.g. *Pecten fumatus*). Scallop fisheries worldwide are well known for being unstable, e.g. 'boom or bust'. Parameters determining colonisation of new substrate and establishment of new beds are poorly known. Beds (colonies of adults) are the targets of dredge fishing. In Bass Strait, the location, extent

and age of beds is closely monitored and harvesting arrangements aim to achieve a balance between socio-economic gain and long-term sustainability of the resource. Beds are thought to have a finite life. Individuals in beds may represent a few same-age cohorts with little or no self-recruitment. The species may have a finite life-span in contrast to some species with indeterminate growth where upper limits on age are not fixed. A molecular biomarker for age and condition would identify beds at the end of their life, whereupon they could be harvested at a more rapid rate.

Variation in telomere length in a cohort of abalone of similar shell size (Section 4.2.3) and among *J. edwardsii* pueruli (Section 4.3.3) was demonstrated here. Some abalone with a shell size of 40-80 mm had longer telomeres than expected, and two of seven pueruli had telomere lengths shorter than expected. Intra-cohort variation in telomere length would be expected if telomere length was associated with life-span. We propose that variation in telomere length within cohorts of the same age could predict the proportion of individuals in a cohort with a shortened lifespan. If so, these proportions could be used to assign a 'condition' factor to a population, which may predict the future survival of that population. This may be relevant to maximising economic returns from fisheries populations, as populations with a low condition factor (i.e. high proportion of short life-span individuals) could be harvested at a higher rate. In this sense, condition factor is a predictor of natural mortality. There is also the potential use of telomere length in the aquaculture industry as a method of selecting longer life-span animals for grow-out or for broodstock.

7. Planned Outcomes

This project was successful in underpinning the use of telomere DNA as a biomarker for age in invertebrate fisheries species.

The most feasible method of estimating telomere length (terminal restriction fragment assay, TRF assay) was comprehensively tested for accuracy, precision and reliability in a cost-benefit framework. Adequate levels of precision and reliability were achieved (Table 11), but some issues were not overcome (e.g. degradation and contamination of DNA). In addition, the monetary and human resource cost of the assay was determined to be high. The 'Further Development' section (above) contains recommendations for the development of alternative methods of estimating telomere length, which should be used in future projects.

This project has clarified the way forward for the use of telomeric DNA as a biomarker for age in fisheries species. The new technology is likely to be most productively applied to mollusc species, with further development needed for its implementation in crustacean species.

Table 11: Criteria that were proposed for 'proof of concept' for the use of telomere DNA as a biomarker for age by the project steering committee in October 2007 compared to the subsequent performance of the project in September 2010.

Criteria	Outcome
Precision (repeatability) is essential. For example, telomere length measurements on the same individual should have variability of about 5%, and tests on animals of the same age should be 20% or less.	Achieved. Coefficient of variation for repeated measurements on the same animal was 1-2% (within gel) and 10-14% (between gel).
Notwithstanding the degree of precision, the correlation statistics on the relationship between telomere length and age should be sufficient to inform the downstream use of the method in fisheries assessments. For example, is the correlation 'useful' in terms of information needed for fisheries stock assessment?	Further research recommended. The species where this is most likely to be achieved is temperate abalone.
Ideally, we need to know how it will work over a range of species.	Achieved. The method appears to have potential as a biomarker for age in molluscs, but not crustaceans.
As an alternative to a correlation between telomere length and age, we could measure telomere length in lobsters that are the same size, but presumably are different in age. However, in this case the lobsters have grown under different environments, which may have indirectly influenced telomere length. Also, telomere length here becomes a proxy for age, and the relationship between telomere length and age will not necessarily be known for lobsters.	Further research recommended. Some indication of systematic telomere length variation among animals in the same size or age cohort. This may lead to the development of telomere length as a biomarker for life expectancy or cumulative lifetime stress.
Aim for 90 points per species for correlation analysis (i.e. three age classes of 30 individuals). Southern blotting method should measure 30 animals per week. Also, will try the Q-PCR method and use if favourable.	Achieved for some (e.g. school prawns) but not all species. Further research recommended as the Q-PCR method remains to be tested.
Could follow telomere length over time in a single individual. To do this we would need to have a non-lethal tissue sampling method, and a way of re-sampling the same animal.	Further research recommended. Non-lethal tissue samples (e.g. blood) were not appropriate for the TRF assay used here (i.e. too little genomic DNA).

8. Conclusions

This project has evaluated and developed techniques for the investigation of telomere length as a biomarker in invertebrate fisheries species. The results across species presented here suggest that the method has greater promise for molluscan, rather than crustacean, species. A clear relationship between shell size and telomere length was demonstrated for temperate abalone and a relationship was apparent in Sydney rock oysters. But, there was little correlation for the crustacean species; school prawns, spanner crabs or two species of lobster (*S. verreauxi* and *J. edwardsi*). The method used to estimate telomere length (TRF assay) was not ideally suited to assay the long telomeres of spanner crabs and lobsters, however telomere lengths of school prawns were in the range where accurate estimates should have been produced. The relationship for abalone, while promising, needs to be tested on samples from other regions and samples re-collected from the same location.

Some information was gathered on factors that may affect the application of telomere length as a biomarker in fisheries populations. Gender did not influence the size of telomeres in school prawns with carapace sizes of 10-25mm. Tissue type was thought to affect telomere length, as tissue that is mitotically active may have shorter telomeres. No consistent pattern between tissue type was observed for eastern rock lobster (*Jasus edwardsii*) and Sydney rock oysters (*Saccostrea glomerata*), but for the vertebrate fish (*Bidyanus bidyanus*) telomere differed in length according the tissue used. The accuracy of a biomarker for age for fisheries populations should be tested across several populations as in Sydney rock oysters (*Saccostrea glomerata*) at least; the relationship appeared to vary between a population in Queensland and one in New South Wales (Table 12).

The TRF method is time-consuming (i.e. 36 samples can be assayed by one person in eleven days) and expensive (i.e. $37 per sample). It is not suitable for assaying the large numbers of individuals required for the analysis of age-structure in wild populations. The development of a Q-PCR method is recommended. The ability to assay abalone telomere length using Q-PCR may lead to data collection as a part of future projects that will provide the information needed to improve the fine-scale, predictive capacity of stock assessment models for sustainable abalone harvest. In turn, this will maximise the economic, social and environmental outcomes of the exploitation of the abalone resource in southern Australia.

Aging using telomeric DNA has the potential to improve the way in which fisheries scientists age populations of fisheries species. This project has enhanced Australia's reputation for excellence in scientific research on fisheries. This will improve Australia's reputation for prudent and profitable exploitation of fisheries stocks, which will flow on to the wider acceptance of Australian seafood worldwide in a market that is becoming more capable of making informed choices about source of product.

Table 12: Overall summary of the relationship between telomere length and age (or size) across the species and the variables that were tested in this project. Data was not obtained in some areas (NA).

	Crustacea				Mollusca		Vertebrate
	School prawn *Metapenaeus macleayi*	Spanner crab *Ranina ranina*	Southern lobster *Jasus edwardsii*	Eastern lobster *Sagmariasus verreauxi*	Sydney rock oyster *Saccostrea glomerata*	Abalone *Haliotis rubra*	Silver perch *Bidyanus bidyanus*
Cohort type	By size	By size	By size	By size	By age	By shell size	By age
Cohort number	Numerous	Two	Two	Four	Four	Numerous	Three
Telomere length (base pairs)	5,000 to 10,000	Up to 20,000	11,000 to 15,000	16,000 to 18,000	7,000 to 8,000	2,000 to 6,000	3,000 to 5,000
Telomere length and age/size	No relationship	Not able to be determined	No relationship in adults	No relationship	Yes, declined	Yes, declined	Yes, declined in older fish
Telomere length and other variable	Gender: For males and females (10-25 mm carapace length), no difference in telomere length	N/A	N/A	Tissue type: In adults, no difference between telomere lengths in gill and muscle.	Environment: Telomeres from oysters in Moreton Bay (QLD) appear to shorten more rapidly than those from oysters in Wallis Lakes (NSW). Tissue type: No consistent pattern for relative size of telomeres in gill compared to mantle tissue.	N/A	Tissue type: Telomere length was longest in muscle, intermediate in liver and shortest in the heart muscle.
Issues	Pronounced telomeric DNA degradation during storage in the lab.	Telomeres may be at limit of resolution of TRF assay method. Other technical issues encounted.	Some evidence that puerulus have shorter (not longer) telomeres than adults, suggesting that telomeres may increase in length with age.	Telomeric DNA degradation occurred during storage in the lab.	Relationship was not stable among regions (e.g. QLD vs. NSW)	Need to confirm relationship exists with age as well as size classes. There were two classes of smaller abalone depending on telomere length, suggesting its role as a biomarker for body condition.	Evidence of increase from one to two year old fish, followed by a decrease from 2-3 year olds.

9. References

Australian Department of Agriculture Fisheries and Forestry (2008) *Australia's Agricultural Industries – At A Glance*. First Edition. Australian Government, Canberra.

Baerlocher, G. M., Mak, J., Roth, A., Rice, K. S. and Lansdorp, P. M. (2003) Telomere shortening in leukocyte subpopulations from baboons. *Journal of Leukocyte Biology* 73: 289-296.

Blackburn, E. H. (2009) Telomeres and telomerase: their implication in human age-related diseases. *Experimental Gerontology* 44: 2.

Blackburn, E. H., Greider, C. W. and Szostak, J. W. (2006) Telomeres and telomerase: the path from maize, Tetrahymena and yeast to human cancer and aging. *Nature Medicine* 12: 1133-1138.

Broderick, D., Ovenden, J. R., Buckworth, R. C., Newman, S. J., Lester, R. J. G. and Welch, D. J. (In press) Genetic population structure of grey mackerel (*Scomberomorus semifasciatus* Macleay, 1883) in northern Australia. *Journal of Fish Biology*.

Brown, I. W., Kirkwood, J., Gaddes, S., Dichmont, C. and Ovenden, J. R. (1999) Population dynamics and management of spanner crabs (*Ranina ranina*) in southern Queensland. Final Report to the Australian Fisheries Research and Development Corporation, Project Number 95/022. Queensland Department of Primary Industries, QO99010, Brisbane (145 pp.).

Brown, I. W., Scandol, J., Mayer, D., Campbell, M., Kondylas, S., McLennan, M., Williams, A., Krusic-Golub, K. and Treloar, T. (2008) *Reducing uncertainty in the assessment of the Australian spanner crab fishery*. Final Report to Australian Fisheries Research and Development Corporation, Project Number 2003/046. Queensland Department of Primary Industries. ISBN 978-0-7345-0394-7.

Buckworth, R. C., Newman, S. J., Ovenden, J. R., Lester, R. J. G. and McPherson, G. R. (2007) *The stock structure of Northern and Western Australian Spanish mackerel*. Final Report to the Australian Fisheries Research and Development Corporation, Project Number 1998/159. Fishery Report Number 88: Fisheries Group, Department of Business Industry and Resource Development, Northern Territory Government, Darwin, Australia.

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

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.

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

Chuang, K., Godwin, R. M., Bennett, M. and Ovenden, J. R. (In preparation) Telomere biology in a freshwater fish, *Bidyanus bidyanus* (silver perch). *Journal of Fish Biology*.

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

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

Estabrooks, S. L. (1999) The telomeres of the bay scallop, *Argopecten irradians* (Lamarck). *Journal of Shellfish Research* 18: 401-404.

Godwin, R. M., Brown, I. W., Montgomery, S., Frusher, S., Green, T. and Ovenden, J. R. (Submitted-a). Telomere dynamics in the Sydney rock oyster (*Saccostrea glomerata*): an investigation of the effects of age, tissue type, location and time of sampling. *Marine Biology.*

Godwin, R. M., Frusher, S., Montgomery, S. and Ovenden, J. R. (Submitted-b) Telomere length and its relationship to age in crustacean species *Metapenaeus macleayi*, *Sagmariasus verreauxi*, *Jasus edwardsii* and *Ranina ranina. ICES Journal of Marine Science*.

Godwin, R. M., Ovenden, J. R. and Frusher, S. (In preparation) Telomere length as a potential biomarker for age in abalone (*Haliotis spp.*).

Goewert, A. E. and Surge, D. (2008) Seasonality and growth patterns using isotope sclerochronology in shells of the Pliocene scallop *Chesapecten madisonius*. *Geo-Marine Letters* 28: 327-338.

Guo, X. M. and Allen, S. K. (1997) Fluorescence in situ hybridization of vertebrate telomere sequence to chromosome ends of the Pacific oyster, *Crassostrea gigas* Thunberg. *Journal of Shellfish Research* 16: 87-89.

Gurney, L. J., Mundy, C. and Porteus, M. C. (2005) Determining age and growth of abalone using stable oxygen isotopes: a tool for fisheries management. *Fisheries Research* 72: 353-360.

Harley, C., Allsopp, R. and Vaziri, H. (1999) UTSWTELORUN Telomere Quantitation, Revised July 1999 MW by Box Position: Background Correction Adjacent to Values. Modified from TELORUN for Excel For Analysis of mean TRF length. v 1.4. Geron Corporation (http://www4.utsouthwestern.edu/cellbio/shay-wright/research/1UTSWTelorunforweb.xls)

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

Hatakeyama, H., Nakamura, K. I., Izumiyama-Shimorriura, N., Ishii, A., Tsuchida, S., Takubo, K. and Ishikawa, N. (2008b) Corrigendum to 'The teleost *Oryzias latipes* shows telomere shortening with age despite considerable telomerase activity throughout life' [*Mech. Ageing Develop*. 129 (2008), 550-557]. *Mechanisms of Ageing and Development* 129: 692-692.

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

Haussmann, M. F., Vleck, C. M. and Nisbet, I. C. T. (2003) Calibrating the telomere clock in common terns, *Sterna hirundo*. *Experimental Gerontology* 38: 787-789.

Keller, N., Del Piero, D. and Longinelli, A. (2002) Isotopic composition, growth rates and biological behaviour of *Chamelea gallina* and *Callista chione* from the Gulf of Trieste (Italy). *Marine Biology* 140: 9-15.

Klapper, W., Kuhne, K., Singh, K. K., Heidorn, K., Parwaresch, R. and Krupp, G. (1998) Longevity of lobsters is linked to ubiquitous telomerase expression. *FEBS Letters* 439: 143-146.

Lang, G. H., Wang, Y., Nomura, N. and Matsumura, M. (2004) Detection of telomerase activity in tissues and primary cultured lymphoid cells of *Penaeus japonicus*. *Marine Biotechnology* 6: 347-354.

Lin, K. W. and Yan, J. (2008) Endings in the middle: Current knowledge of interstitial telomeric sequences. *Mutation Research-Reviews in Mutation Research* 658: 95-110.

Lu, T., Pan, Y., Kao, S. Y., Li, C., Kohane, I., Chan, J. and Yankner, B. A. (2004) Gene regulation and DNA damage in the ageing human brain. *Nature* 429: 883-891.

McHale, M., Broderick, D., Ovenden, J. R. and Lanyon, J. M. (2008) A PCR assay for gender assignment in dugong (*Dugong dugon*) and West Indian manatee (*Trichechus manatus*). *Molecular Ecology Resources* 8: 669-670.

McShane, P. E., Smith, M. G. and Beinssen, K. H. H. (1988) Growth and morphometry in abalone (*Haliotis rubra* Leach) from Victoria. *Australian Journal of Marine and Freshwater Research* 39: 161-166.

Miller, K. J., Maynard, B. T. and Mundy, C. N. (2009) Genetic diversity and gene flow in collapsed and healthy abalone fisheries. *Molecular Ecology* 18: 200-211.

Miller, S. A., Dykes, D. D. and Polesky, H. F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research* 16: 1215-1215.

Monaghan, P. (2010) Telomeres and life histories: the long and the short of it. *Annals of the New York Academy of Sciences* 1206: 130-142.

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

Naylor, J. R., Manighetti, B. M., Neil, H. L. and Kim, S. W. (2007) Validated estimation of growth and age in the New Zealand abalone *Haliotis iris* using stable oxygen isotopes. *Marine and Freshwater Research* 58: 354-362.

Nell, J. (2001) The history of oyster farming in Australia. *Marine Fisheries Review* 63: 14-25.

O'Connor, W. A. and Dove, M. C. (2009) The changing face of oyster culture in New South Wales, Australia. *Journal of Shellfish Research* 28: 803-811.

Ovenden, J., Peel, D., Street, R., Courtney, A., Hoyle, S., Peel, S. and Podlich, H. (2007) The genetic effective and adult census size of an Australian population of tiger prawns (*Penaeus esculentus*). *Molecular Ecology* 16: 127-138.

Ovenden, J. R., Kashiwagi, T., Broderick, D., Giles, J. and Salini, J. P. (2009) The extent of population genetic subdivision differs among four co-distributed shark species in the Indo-Australian archipelago. *BMC Evolutionary Biology* 9: 40.

Plohl, M., Prats, E., Martínez-Lage, A., González-Tizón, A., Méndez, J. and Cornudella, L. (2002) Telomeric localization of the vertebrate-type hexamer repeat, (TTAGGG)n, in the wedgeshell clam *Donax trunculus* and other marine invertebrate genomes. *Journal of Biological Chemistry* 277: 19839-19846.

Sahara, K., Marec, F. and Traut, W. (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. *Chromosome Research* 7: 449-460.

Sakai, M., Okumura, S. I. and Yamamori, K. (2005) Telomere analysis of Pacific abalone *Haliotis discus hanna*i chromosomes by fluorescence in situ hybridization. *Journal of Shellfish Research* 24: 1149-1151.

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

Sambrook, J. and Russell, D. (2001) *Molecular Cloning. A laboratory manual*. Third Edition. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.

Sheehy, M. (1990) Potential of morphological lipofuscin age-pigment as an index of crustacean age. *Marine Biology* 107: 439-442.

Shepherd, S. A., Alwahaibi, D. and Alazri, A. R. (1995) Shell growth checks and growth of the Omani abalone *Haliotis mariae*. *Marine and Freshwater Research* 46: 575-582.

Siddeek, M. S. M. and Johnson, D. W. (1997) Growth parameter estimates for Omani abalone (*Haliotis mariae*, Wood 1828) using length-frequency data. *Fisheries Research* 31: 169-188.

Stevick, P. T., Palsboll, P. J., Smith, T. D., Bravington, M. V. and Hammond, P. S. (2001) Errors in identification using natural markings: rates, sources and effects on capture-recapture estimates of abundance. *Canadian Journal of Fisheries and Aquatic Science* 58: 1861-1870.

Sumpton, W. D., Ovenden, J. R., Keenan, C. P. and Street, R. (2008) Evidence for a stock discontinuity of snapper (*Pagrus auratus*) on the East coast of Australia. *Fisheries Research* 94: 92-98.

Sumpton, W. D., Sawynok, B. and Carstens, N. (2003) Localised movement of snapper *(Pagrus auratus*, Sparidae) in a large subtropical marine embayment. *Marine and Freshwater Research* 54: 923-930.

Tarr, R. J. Q. (1995) Growth and movement of the South African abalone *Haliotis midae* – a reassessment. *Marine and Freshwater Research* 46: 583-590.

Vitkova, M., Kral, J., Traut, W., Zrzavy, J. and Marec, F. (2005) The evolutionary origin of insect telomeric repeats, (TTAGG)(n). *Chromosome Research* 13: 145-156.

Vleck, C. M., Haussmann, M. F. and Vleck, D. (2003) The natural history of telomeres: tools for aging animals and exploring the aging process. *Experimental Gerontology* 38: 791-795.

Ying, T. C. (2005) *Evaluation of telomere length as an age-marker in marine species*. Master of Philosophy thesis, University of Hong Kong.

Appendix 1 – Intellectual Property

This research has been placed in the public domain.

Appendix 2 – Project Personnel

Dr Jenny Ovenden	Principal Research Scientist	Queensland Government employee
Dr Rosie Godwin	Research Scientist	Employed by Project (full-time)
Ms. Majorie D'Sousa	Laboratory Technician	Employed by Project (part-time)
Mr. Vivek Mitter	Laboratory Technician	Employed by Project (part-time)
Ms. Kendy Chuang	Laboratory Technician	Employed by Project (casual)

Appendix 3 – Raw Data on eResearch Archive

Raw data is available at http://www2.dpi.qld.gov.au/extra/era/index.html
Search database using key words.

Appendix 4 – Tissue Samples Collected During the Project

A total of 1,425 individuals were collected across thirteen species.

Taxonomic Group	Species	Source	Sample details	Known age (A) or size (S)?	Age/Size range of samples	Approx sample size per cohort	DNA extracted?
Mollusc	Sydney rock oyster *Saccostrea glomerata*	Jane Clout (Moreton Bay) Anthony Sciacca (Wallis Lake)	Moreton Bay: 27 approx. 1 yr old. 40 approx. 3 yrs old } 2007 35 approx. 4 yrs old Four tissue types taken from 3-4 year-olds: mantle, gills, adductor muscle and digestive gland; and whole sac containing all tissues from 1 year-olds. Ten oysters each aged two months, and approx. 1 year and 2 years. Sampled in 2009. Wallis Lake: 40 approx. 18 months old 25 approx. 1 year old } 2009 25 approx. 3 years old 25 approx. 4 years old Three tissue types taken from 3-4 year-olds: mantle, gills, adductor muscle; and whole sac containing all tissues from 1 year-olds.	A	2 months to 4 years	10-40	Y
Mollusc	Tropical abalone *Haliotis asinina*	Bernie Degnan	10 individuals 11 months old (whole animal); and 10 individuals 18 months old. Muscle tissue from foot sampled.	A	11-18 months	10	Y
Mollusc	Temperate abalone Black lip (*H.rubra*) x green lip (*H. laevigata*) hybrids and wild black lip (*H. rubra*)	Stewart Frusher	*H.rubra* x *H. laevigata* 8 individuals each 2, 3 and 4 years old from a commercial aquaculture facility. *H.rubra* wild caught age unknown, range of sizes instead 15 large 140-155 mm. 30 variety of sizes 20-85mm shell.	A S	2-4 years 20-155 mm shell size	8	Y

Taxonomic Group	Species	Source	Sample details	Known age (A) or size (S)?	Age/Size range of samples	Approx sample size per cohort	DNA extracted?
Crustacean	Spanner crab *Ranina ranina*	Ian Brown, QDPI&F	130 wild crabs approx. 40 of each size class; small (less than 85 mm carapace width), medium (85 to 114 mm) and large (greater than 115 mm).	S	Carapace 80-140mm	40	Y
Crustacean	Mud crab *Scylla serrata*	Brian Patterson, BIARC	30 from feed trials (15 from trial A and 15 from trial D), all approx. 4 months old. Crab carapace length was recorded, muscle, hepatopancreas and gills kept for all samples.	A	4 months	15	N
		Mark Grubert, NT Fisheries	Three tissues from each animal (brain, hepatopancreas, muscle) in ethanol. Eleven age classes (sample size); 83 days (6), 116 days (6), 143 days (6), 170 days (6), 206 days (9), 207 days (5), 241 days (6), 262 (6), 360 (6), 366 (8), 402 (6). Sexes known.	A	83-402 days	6	N
Crustacean	Eastern rock lobster *Sagmariasus verreauxi*	Geoff Liggins, Steve Montgomery, NSW DPI	5 large size classes – carapace length 103-130 mm, purchased live from Charis Seafoods, Southport (sampled heart gill muscle and antennal gland). NSW DPI&F (Steve Montgomery) supplied 30 pueruli (newly settled), carapace length 11-15 mm and 30 animals approx. 3 years old – small size class, carapace length 65-87 mm (sampled muscle tissue only). 6 very large size class, carapace length 150-159 mm (heart, gill and muscle tissue taken), commercially purchased from Coffs Harbour.	A and S	Puerulus to very large	5-30	Y
Crustacean	Southern rock lobster *Jasus edwardsii*	Stewart Frusher, TAFI	31 puerulus samples and 42 older individuals (age unknown), muscle and digestive gland samples. Glenn Dunshea also sent us tissue samples of 17 adults of varying size (blood, muscle tissues also ovary of some individuals).	S/A	Puerulus and adult	35	N
Crustacean	School prawn *Metapenaeus macleayi*	Steve Montgomery, NSW DPI	Wild, from beam trawlers, Grafton, Clarence River. 150 individuals sampled. Three size classes; small (10-14 mm carapace length), medium (16-23 mm) and large (16-25 mm); with 25 males and 25 females in each size class. Muscle tissue only collected. Small and medium	S	10-25 mm carapace length	25	Y

Taxonomic Group	Species	Source	Sample details	Known age (A) or size (S)?	Age/Size range of samples	Approx sample size per cohort	DNA extracted?
			individuals taken from the lake and large individuals taken from the ocean.				
Crustacean	Banana prawn *Fenneropenaeus merguiensis*	Paul Palmer, BIARC	Cultured prawns from BIARC 52 approx. 2 months old – bodies were put into 1 tube and heads in another. 50 approx. 7 months old (muscle tissue only)	A	2-7 months	50	N
Crustacean	Giant tiger prawn *Penaeus monodon*	Tom Dixon, CSIRO	80 young individuals, approx. 2 months old	A	2 months old	80	N
Crustacean	Red Claw *Cherax quadricarinatus*	Evizel Seymour, QDPI Walkamin	Only Muscle tissue was sampled from Red Claw. Individuals were all from the same hatching. 53 individuals approx. 2½ months old. NB: The animals had died after delivery and some hours before sampling therefore we received a second, repeat batch of 60 individuals, received three weeks later (approx. 3 months old). 63 approx. 9 months old 55 approx. 15 months old 50 approx. 19 months	A	3-19 months	50	N
Fish	Whiting *Sillago cillata*	Paul Palmer, BIARC	20 whiting approx. 60 days old, whole bodies placed in tubes. 30 whiting 7 months old (muscle tissue sampled).	A	Will have 3	25	N
Fish	Silver Perch *Bidyanus bidyanus*	Steve Montgomery, NSW DPI	Four age classes: 2008: 3 month old, 1 and 2 year-olds sampled in from freshwater hatchery, Grafton, NSW. 30 individuals sampled from each size class. Wherever possible heart, muscle and liver tissues were taken. 2009: 3 year-old fish heart, liver, muscle, ootilith and fin – 10 individuals sampled.	A	3 months to 3 years	10-30	Y

Appendix 5 – Scientific Manuscripts Submitted for Publication

Appendix 5.1 – Submitted Manuscript

Telomere dynamics in the Sydney rock oyster (*Saccostrea glomerata*): an investigation of the effects of age, tissue type, location and time of sampling

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Abstract

Telomere length has been purported as a biomarker for age and could offer a non-lethal method for determining the age of wild-caught individuals. Molluscs, including oysters and abalone, are the basis of important fisheries globally and have been problematic to accurately age. To determine if telomere length could provide an alternative means of ageing molluscs, we evaluated the relationship between telomere length and age using the commercially important Sydney rock oyster *(Saccostrea glomerata)*. Telomere lengths were estimated from tissues of known age individuals from different age classes, locations and at different sampling times. Telomere length generally decreased across the tested age range (two months to four years) and the relationship was similar amongst tissue types. Regional and temporal differences in telomere attrition rates were also observed. The relationship between telomere length and age was weak, however, with individuals of identical age varying significantly in their telomere length making it an imprecise age biomarker in oysters.

Introduction

Information about the ages of individuals is needed to estimate the age structure of a population. This gives insights into the growth rates, mortality and productivity of the population and so is an integral component of sustainable fisheries management. Techniques for collecting information about the ages of fish resources have been well documented and extensively evaluated but are still susceptible to errors (Campana, 2001). Inaccurate age estimations when applied to fisheries management can have serious consequences, leading to over exploitation of a collapse of populations or species as exampled by Campana (2001).

Age and growth information of mollusc species has also been problematic with a range of techniques applied including tag-recapture methods (McShane *et al.* 1988), counting growth rings of shells, studying the ratio of isotopes in the carbonate of shells (Keller *et al.* 2002; Gurney *et al.* 2005; Naylor *et al.* 2007; Goewert and Surge, 2008) and cohort or length frequency analyses (Shepherd *et al.* 1995; Tarr, 1995; Siddeek and Johnson, 1997). Although these methods have provided useful information, they tend to be species specific, time consuming, labour intensive, require careful validation and are often deleterious or lethal to the organism. The development of a DNA-based method for estimating the age of shellfish may

provide an alternative to traditional methods and could be more direct, less expensive, quicker and could be performed non-lethally.

Telomeres are ribonucleoprotein structures at the ends of linear chromosomes composed of short, tandem, highly repeated DNA sequences and associated proteins. Their main function is to protect the ends of the chromosomes from degradation and hence to provide genome stability (Szostak and Blackburn, 1982; Blackburn, 1991). Haussmann and Vleck (2002) and others (Tsuji *et al.* 2002; Haussmann *et al.* 2003; Vleck *et al*. 2003) suggested that the telomeric DNA at the ends of chromosomes could provide a tool for aging animals since telomeric DNA of somatic cells normally shortens with progressive cell divisions over an animal's life-span due to the 'end-replication problem' (Watson, 1972). A significant correlation has been observed between age and telomere length for humans (von Zglinicki and Martin-Ruiz, 2005), some species of birds (Haussmann and Vleck, 2002; Haussmann *et al.* 2003; Vleck *et al.* 2003) and fish (Nakagawa *et al.* 2004; Ying, 2005; Hatakeyama *et al.* 2008; Hartmann *et al.* 2009).

In this study the terminal restriction fragment (TRF) assay was used to determine if a relationship between telomere length and age exists in oysters. This robust method is appropriate for species with limited genome resources available, to estimate telomere length from individuals when the amounts of available tissue are not limiting. However, if a strong relationship is detected it would be desirable to develop a high throughput, quantitative PCR method which would allow non-lethal sampling from very small tissue samples such as biopsies of skin or muscle, or from hemolymph. This sample could also be used for genetic stock identification and gender assays.

The telomere repeat sequence TTAGGG has been described for a number of bivalve molluscs, such as Pacific oyster (*Crassostrea gigas*) (Guo and Allen, 1997), bay scallop (*Argopecten irradians*), blue mussel (*Mytilus galloprovincialis*), wedgeshell clam (*Donax trunculus*) (Plohl *et al.* 2002) and sand scallop (*Euvola ziczac*) (Owen *et al.* 2007). Telomerase is an important enzyme that counteracts telomere attrition (Greider and Blackburn, 1985; 1989). It has been found to be active in lobsters (Klapper *et al.* 1998b), sea urchins (Francis *et al.* 2006), and some bivalves (Owen *et al.* 2007) and it has been suggested that sustained telomerase activity is necessary in species with indeterminate growth or high regenerative potential to maintain telomeres throughout their lifespan (Klapper *et al.* 1998a; 1998b). In animals exhibiting telomere ubiquitous telomerase expression or other maintenance mechanisms it is possible that little association between telomere length and age might be found.

The Sydney rock oyster (*Saccostrea glomerata*) (formerly *S. commercialis*) is a highly valuable seafood resource in Australia. It is endemic from 19°S-37°S on the Australian east coast (Nell, 2001; O'Connor and Dove, 2009) but also occurs on Australia's northern and western coastlines and is also endemic in New Zealand. Oyster farming occurs along the east coast of Australia in the mid to lower part of inter-tidal zone of marine estuaries and bays.

Sarccostrea glomerata was selected as a model species to test for a relationship between telomere length and age in a mollusc species because of its distribution over a wide latitudinal range and ready access to individuals of known age. We hypothesised the relationship between telomere length and age would be significant in at least one tissue type and consistent between different environments. Additionally, we tested if the relationship was repeatable in one environment through time. These characteristics are essential for telomere length to be a satisfactory biomarker of age.

This paper also examines sources and effects of experimental variation on the estimation of the telomere length using the TRF assay. This work is part of a larger study to determine whether telomeric analyses offer a new, cost effective method for collecting biological information on aquatic species, particularly those that are difficult to age using conventional techniques.

Methods

Sampling of tissues

The relationship between telomere length and age in *S. glomerata* was examined by studying cultured oysters of known age in two different regions and by studying oysters from a single location sampled at two different times. Farmed oysters are usually marketed at three to four years of age, thus older oysters of known age were unavailable for the study. The age classes studied were largely governed by the availability of oysters at the time of sampling.

The study encompassed three experiments. The aim of experiment 1 was to assess the relationship between telomere length and age in oysters from one location, Moreton Bay, Queensland, Australia (27°20'S, 153°23'E). Oysters known to be one, three and four years of age were obtained in 2007. Telomere length estimates were made on

individuals from the three age classes using gill and mantle tissue.

In experiment 2 we examined the relationship between telomere length and age in another location, Wallis Lake, Tuncurry, New South Wales, Australia (32°11'S, 152°28'E), which is approximately 700 km and 5° of latitude south of Moreton Bay. Oysters aged one, three and four years were obtained from a farm in 2009. One-year-old oysters were removed from their shells and sampled whole owing to their small size but gill and mantle tissue was dissected from three and four year old individuals. Additionally, oysters of 1.5 years of age were obtained from this location six months later since the availability of one-year-old oysters was limited. Gill tissue only was used for telomere length estimates of 1.5 year old oysters.

Experiment 3 assessed temporal effects on the relationship between telomere length and age at a single location, Moreton Bay. A second sampling was conducted at the Moreton Bay farm in 2009, two years after the first sampling. Oysters aged two months, one year and two years were available for testing and gill tissue only was used. This was because DNA extracted from mantle had previously been more susceptible to degradation than DNA from gill tissue and therefore additional effort was made to separate the gill tissue away from the remaining tissues even in two month old oysters.

Moreton Bay oysters were derived from hatchery produced spat whereas Wallis Lake oysters originated from natural caught spat. At the laboratory live oysters were opened and their tissues dissected as outlined above. Tissue samples were placed in 2 mL cryotubes, snap frozen, and then transferred immediately to a -80°C freezer for storage.

Extraction of total genomic DNA and TRF assays

The TRF assay provides an estimate of mean telomere length per individual. High molecular weight genomic DNA was extracted from frozen tissue using the standard method of Sambrook and Russell (2001). This involved cell lysis in the presence of EDTA, sodium dodecyl sulphate (SDS) and proteinase K followed by phenol/chloroform extractions and ethanol precipitation. The resulting DNA was redissolved in TE (Tris-EDTA) buffer pH 8.0, 1 mM DTT (Dithiothreitol) to minimise degradation and oxidation of the telomeric DNA. A range of TRF assays was conducted on oysters using genomic DNA from mantle, gill or whole animal.

The TRF assays were performed using the Telo*TAGGG* Telomere Length Assay kit (Roche Diagnostics Australia Pty. Ltd, Castle Hill, NSW, Australia) with the following specifications and modifications. The telomere repeat sequence of *S. glomerata* was assumed to be TTAGGG as reported for other molluscs (Guo and Allen, 1997; Estabrooks, 1999; Plohl *et al.* 2002; Sakai *et al.* 2005) and hybridisation of the probe to oyster genomic DNA was confirmed by dot blot (data not shown).

Prior to each set of TRF assays the quantity and integrity of genomic DNA extracts were checked by gel electrophoresis. Genomic DNA (approximately 2 µg) was digested with *Hinf*I and *Rsa*I for three hours at 37°C and separated using conventional gel electrophoresis on a 1 x TAE 0.8% agarose gel at 35 V for 20 hours at 4°C. Samples were loaded in randomised order on every gel to minimise within-gel effects. Typically, six individuals were used per tissue per age class making a total of 18 samples per TRF assay. Each assay was repeated on three occasions. To provide controls, three Moreton Bay oysters were included in assays with the Wallis Lake oysters.

DNA was denatured prior to transfer to nylon membrane and subsequent transfer, hybridisation, chemiluminescence and detection were according to the Roche Telo*TAGGG* kit instructions. Chemiluminescent signals from the membrane were captured on x-ray film using multiple exposures as appropriate.

Determination of telomere length

Estimates of telomere length were obtained by comparing the telomere smear to a molecular size standard. X-ray films were scanned using a high definition scanner and uploaded into Quantity One v 4.2.3 imaging software (Biorad, Gladesville, NSW, Australia) for analysis. An example of an autoradiograph obtained from an oyster TRF assay is shown in Figure 1. The telomere smear was visible in the top half of each lane and below each smear was a pattern of bands that appeared to vary between individuals. This banding pattern was assumed to be associated with interstitial and sub-telomeric (IST) repeats.

Mean TRF measurements were determined by the method outlined in the Roche Telo*TTAGG* Telomere Length Assay Protocol. In summary, each lane was overlaid with a grid of 25 equal-sized boxes, which spanned the lane from above the largest molecular weight marker (23,130 bp) and excluded bands associated with the IST DNA (Figure 1). The total intensity data inside in each box and molecular weight at the centre of each box was estimated using Quantity One software

(Biorad). A minimum background subtraction as suggested in the Roche protocol was applied in each lane whereby the box with the lowest signal volume was subtracted from each of the remaining 24 boxes in each lane.

Telomere length for an individual oyster was calculated from the TRF assay using the formula

$$\text{Mean telomere length} = \Sigma (OD_i)/ \Sigma (OD_i/L_i)$$

where OD is the total chemiluminescent signal in each box and L_i is the length of the TRF fragment at the position 'i'. This formula takes into account the higher signal intensity from larger TRFs due to multiple hybridisations of the telomere specific hybridisation probe.

Estimation of experimental errors

Effect of x-ray saturation on telomere length estimates

The relative quantity of restriction fragments, and hence overall mean telomere length, can be incorrect if saturation occurs in the x-ray film at high signal intensities. Saturation occurs when the x-ray film is no longer capable of registering more incident radiation, regardless of the intensity of the signal. The effect of varying film exposure times on telomere length calculation was examined for 18 samples by exposing their chemiluminescent signals to a series of x-ray films for 30 seconds, one, two and five minutes. Mean telomere length was then calculated on the 18 samples for each of the four exposures.

Effect of relative versus fixed grid-box positioning on telomere length calculation

Two methods of orientating the 25-box grid on each lane were tested to see which gave the most repeatable telomere length measurements.

i. Relative box positioning whereby the grid of boxes was placed to include the telomeric smear but to exclude most, if not all of the very bright low molecular weight bands associated with IST bands.

ii. Fixed box positioning whereby the grid of boxes was positioned identically on every lane with the lower edge aligned to the 4361 molecular weight marker (Figure 1). This ensured that the telomeric smear was encompassed by the box grid but most of the IST bands were excluded from the overall measurement.

TRF assays were conducted on 18 individual oysters in three replicate experiments. Telomere length estimates were made from each of resulting three autoradiographs using first the relative box positioning method and then the fixed box positioning method as described above.

Assessment of the variation in estimates between and within replicate experiments

Estimates of between-experiment coefficients of variation were made by repeating samples across at least three gels.

Genetic comparisons

Variation in the shape of the relationship between age and telomere length between oysters collected from the same farm at different times may be explained by genetic differences between cohorts. To test this, the degree of IST band dissimilarity between pairs of individuals from the same and different cohorts was used as a surrogate for genetic relatedness, in the same way that AFLP markers are used to assess genetic diversity and population structure (Bonin *et al.* 2007).

Oysters from 2007 and 2009 cohorts from the Moreton Bay farm were compared; 18 from the 2007 group and 14 from the 2009 group. The presence or absence of IST bands in 16 bins (each 200 bp) ranging 1.7-5 kb were scored for the 32 oysters.

The presence or absence of IST bands between pairs of individuals was expressed as number of pairwise differences and was calculated using Arlequin v 3.1 (Schneider *et al.* 2000). The pairwise dissimilarity matrix generated by Arlequin was then analysed using MEGA v 4 software (Tamura *et al.* 2007) to calculate the average number of pairwise differences within and between cohorts and to assess phylogenetic relationships. The phylogenetic relationship was inferred using the minimum evolution method (Rzhetsky and Nei, 1992).

Data Analysis

All data were subjected to analysis of variance (ANOVA) using GenStat (Payne *et al.* 2009). The individual oysters were taken as the independent experimental units, with split-plots adopted for the different tissues (when appropriate) and also for the replicate measurements of the same individuals. Telomere length was the dependent variable, with age groups and tissue types being the independent factors for these analyses. Inspection of residual plots indicated that no transformation of the data was necessary. Least-significance difference (LSD) testing was applied when the ANOVA indicated a significant ($P<0.05$) difference. In some cases, unpaired Student's

t-tests were used to compare means of different age classes.

For experiment 1 data, obtained from 2007 oysters from Moreton Bay, the ANOVA terms were age, tissue and their interaction. Experiment 2 did not contain a complete data set for every age class for gill and mantle tissue. Therefore the effect of age on telomere length was analysed separately for each tissue using a one-way design. For gill tissue the 1.5, three and four-year-old age classes were compared, whereas for mantle tissue, only the three and four-year-old age classes were compared. Telomere lengths of one-year-olds were estimated from whole bodies containing a mixture of tissues. Therefore these data were not included in ANOVAs for individual tissues.

In experiment 3, the effect of age on telomere length was examined in the second sampling of oysters from Moreton Bay in 2009, using a one-way design with data obtained from gill tissue.

Results

Effect of experimental variation on telomere length estimates using the TRF assay

Effect of x-ray saturation on telomere length estimates

Exposure time significantly affected telomere lengths estimates of given individuals whereby estimates of all 18 test samples decreased as exposure time increased (ANOVA $F_{(3,42)}$ = 21.66, P<0.001). Therefore exposure time needs to be carefully controlled for accurate and repeatable estimates of telomere length to be made. An example of how exposure time affects telomere length estimates is shown in Figure 2. If exposure time is too short, the chemiluminescent signal from the telomere fragments (particularly the shorter lengths) are not registered on the film. This causes the mean telomere length to be overestimated. However, for longer exposure times, the film becomes progressively more saturated by the signal and consequently there is a loss in sensitivity, resulting in underestimation of mean telomere length. To ensure the most accurate telomere length, estimates were made per oyster; a graph of signal intensity per lane was examined for a range of x-ray exposure times. The lane with maximum signal intensity but minimum saturation was selected qualitatively for telomere length measurement for each sample in this study.

Effect of relative versus fixed grid-box positioning on telomere length estimation

Both grid-box positioning methods tested in this study permitted accurate estimates of telomere length. There was no significant difference between the two methods (ANOVA $F_{(1,35)}$ = 0.13, P=0.720). The overall mean for the 18 test samples was 9,007 bp for the variable box-positioning and 8,973 bp for fixed box positioning. There was a coefficient of variation (CV) of 4.4% between the methods and 12% between the replicate experiments for each method. The average standard deviation between the three replicate experiments irrespective of grid-box positioning method was 955 bp.

Assessment of the variation in estimates between replicate experiments

Coefficients of variation between replicate experiments of oysters were between 10 and 14%. Within-gel effects are likely to be smaller than this. Although the within-gel effects were not tested for oysters, preliminary experiments in our laboratory with a range of other mollusc and crustacean species indicated that CVs between repeated measurements within one experiment (i.e. for a replicate measurement of a single individual run three times on one gel) were 1-3% (data not shown).

Experiment 1: The effect of age and tissue type on telomere length of Moreton Bay oysters

The average telomere length of oysters sampled from Moreton Bay in 2007 was 9,007 bp. Estimates among the sampled individuals varied from 5,306 to 14,541 bp.

The effect of age on telomere length for both gill and mantle tissue was just outside the P=0.05 level of significance (ANOVA $F_{(2,15)}$ = 2.97, P=0.08) providing reasonable evidence that a relationship between telomere length and age may exist. Unpaired t-tests showed that one-year-old oysters had significantly longer telomeres, based on gill tissue, than three-year-olds (t_{10} = 2.30, P=0.04). In mantle tissue, telomere lengths of one-year-old oysters were significantly different to both three (t_{10} = 2.98, P=0.01) and four-year-old oysters (t_{10} = 2.75, P=0.02). The difference in telomere length between three and four-year-olds was not significant for either tissue (Table 1). The telomeres of one-year-old oysters were approximately 2,400 bp longer than those of three-year-olds suggesting an attrition rate of around 1,200 bp y^{-1} between one and three years of age.

The pattern of decrease in telomere length with age was similar for each tissue type (Table 1) although telomere lengths of gill tissue were consistently longer than those of mantle tissue (ANOVA $F_{(1,15)}$ = 4.84, P=0.04). Mean telomere lengths estimated from gill and mantle tissue across all individual in all age groups were 9,295 and 8,719 bp, respectively.

Experiment 2: The relationship between telomere length, age and tissue in a second environment

Regional differences in the relationship between telomere length and age were investigated using farmed oysters of ages one, three and four years from Wallis Lake, Tuncurry, New South Wales.

The average telomere length of oysters from Wallis Lake was 7,873 bp (approximately 13% lower than Moreton Bay). Estimates among the sampled individuals varied from 4,920 to 12,995 bp.

Data from one-year-old oysters from Wallis Lake were excluded from comparisons between age classes because tissue types were not separated. One-year-old oysters from Wallis Lake were sampled whole and therefore telomere lengths were estimated from a mixture of tissues including gill, mantle tissue, adductor muscle, digestive gland and stomach tissue. A mean telomere length of 7,785 bp was estimated for one-year-old individuals (n= 10), which was approximately 2,700 bp (25%) lower than that estimated from gill and mantle tissue for one-year-old Moreton Bay oysters.

Though there was a trend for telomere length from gill tissue to decrease with age for Wallis Lake oysters, differences between the age group means were smaller than at Moreton Bay and were non-significant (ANOVA $F_{2,22} = 1.57$, P=0.23). Telomere lengths of three and four-year-old Wallis Lake oysters were comparable to those of similar aged Moreton Bay oysters with age group means being in the range of approximately 6,700-8,800 bp (Table 1). However, estimates of telomere length from 1.5 year-old oysters at Wallis Lake were over 1,500 bp lower than one-year-old oysters at Moreton Bay.

The difference in telomere length between three and four-year-old oysters from Wallis Lake based on mantle tissue was also non-significant. Telomere length estimates were only obtained from mantle tissue of three and four-year-old Wallis Lake oysters and not from 1.5 year-old oysters (Table 1).

Experiment 3: Temporal effects on the relationship between telomere length and age in Moreton Bay

Telomere lengths were estimated from gill tissue of a second sample of Moreton Bay oysters collected in 2009: two months old, one and two years old (Table 2). Age had a significant effect on the telomere length for the three age groups sampled in 2009 (ANOVA $F_{2,25} = 16.69$, P <0.001), with the mean for two-month-old oysters (8,892 bp) being greater than one (5,918 bp) and two-year-old oysters (5,672 bp). Telomere lengths of one and two-year-old oysters did not differ significantly from one another (Fisher's LSD = 1277, P=0.05).

Telomere lengths for age classes from the 2007 and 2009 samples from Moreton Bay were not consistent between temporal re-samplings (Figure 3). Telomere lengths recorded for two-month-old oysters in 2009 were similar to those recorded for the one-year-old oysters collected in 2007. If telomere attrition rates and telomere lengths in larvae were constant between sampling events, younger oysters should have longer telomeres than older oysters. However, telomeres for the one and two-year-old oysters in 2009 were on average 2,000 bp shorter than telomeres of three and four-year-old oysters assayed in 2007 (Table 2). Telomere length tended to decrease across the age groups for both the 2007 and 2009 samples. In general, the telomere lengths of the 2009 sample group were shorter than the 2007 group (Figure 3).

Generally, the variability within a given age class was similar for the 2007 and 2009 samples (Figure 3). The coefficient of variation amongst individuals in the 2007 sample population (across three age cohorts) was 39% compared to 35% for the 2009 group. Most of the variation among individuals in the 2009 sample was amongst the two-month-old oysters (Figure 3).

Genetic comparison between 2007 and 2009 Moreton Bay oysters

There was some evidence that the lack of consistency in telomere length relationships among oysters sampled in 2007 and 2009 were due to genetic differences between cohorts, implying 2007 and 2009 oysters were from different strains or broodstock. IST banding patterns were observed at lower molecular weights in all individuals (Figure 1) and there was evidence for genetic dissimilarity between 2007 and 2009 samples based on these IST bands. The dissimilarity in IST banding patterns between 2007 and 2009 was greater than the mean within-year dissimilarity. The overall average number of pairwise differences in IST banding patterns between the 2007 and 2009 Moreton Bay oysters was 4.83 whereas the average number of pairwise differences within each cohort was 4.31 and 4.75 for 2007 and 2009, respectively. The net number of pairwise differences between the 2007 and 2009 cohorts was 0.64.

Discussion

Effect of experimental variation on telomere length estimates in oysters

Some of the sources of error associated with using the TRF assay to estimate telomere length in *S. glomerata* were evaluated in this study. From these evaluations, we believe reliable estimates of telomere length may be made with this method if sources of error are carefully controlled by good experimental procedure and standardised techniques.

Researchers in molecular ecology or population biology laboratories may have less sophisticated equipment compared to molecular genetics or biotechnology laboratories, where the majority of telomere research in performed. If this were the case, it is especially important to standardise all aspects of the TRF assay from DNA extraction and digestion, electrophoretic conditions, transfer, hybridisation and detection conditions to analysis of autoradiographs in population or ecology laboratories. Conventional electrophoresis equipment was used here for estimating telomere lengths of *S. glomerata* since their telomeres were less than 20 kb, which is the upper resolution limit of conventional apparatus. Accuracy and precision would need to be assessed if conventional equipment was used to assay telomere length using the TRF method for species whose telomere length approached this upper limit.

Exposure time needs to be carefully controlled if accurate and repeatable estimates of telomere length are to be made from chemiluminescent signals captured on x-ray film. Though x-ray film is sensitive for capturing chemiluminescent signals, the response curve of the film is sigmoidal of which the linear region is narrow therefore limiting its dynamic range at 1:100 (Bio-Rad Laboratories Pty. Ltd., Hercules, CA, USA; Protocol Guide, 2008, p. 15; www.biotechniques.com/protocol). This means that saturation is quickly reached and the degree to which the exposure is beyond saturation is not indicated. Therefore a broad range of signals appear alike preventing direct quantitative comparisons.

We have shown here that under-exposed x-ray film will result in an over-estimate of mean telomere length since large fragments are brighter. Over-exposed images will lead to an underestimate of mean telomere length since the signals from large fragments become saturated more quickly with longer exposures (Figure 2). Consequently, it is of utmost importance to choose the correct exposure time and to apply this consistently across a study. Ensuring equal amounts of DNA are loaded in each lane facilitates the choice of the correct exposure time for a group of samples on the same gel. Direct imaging systems which utilise charge-coupled device (CCD) cameras, to capture chemiluminescent signals would have improved the accuracy of telomere length estimates since chemilinescent signals can be captured over a dynamic range of 1:10 000 and the response curve is linear over the whole range (Martin and Bronstein, 1994). The influence of exposure time and variations in DNA loading on telomere length measurements would be greatly minimised. Unfortunately direct imaging equipment, though standard in many laboratories is still not universal.

The genome of *S. glomerata* seems to contain a significant portion of satellite $(TTAGGG)_n$ DNA (i.e. IST bands), which appear to vary between individuals (Figure 2). Some of these bands were close to the bottom of the telomere smear and inclusion of these bright, lower molecular weight bands in estimates of telomere length has the effect of skewing mean estimates downwards. Therefore, when estimating telomere length in species with IST bands, it is essential to stipulate the molecular weight range of fragments that are included in telomere length estimates.

Differences in telomere length between groups of *S. glomerata* samples would need to be greater than ten percent and sample sizes would need to be adequate to be yield statistically significant results using the TRF assay.

Comparison of telomeres in oysters with other mollusc species

Like other mollucs, *S. glomerata* have the telomeric repeat sequence TTAGGG, however telomere lengths vary substantially between mollusc species. Telomere lengths of 0.75-2.3 kb have been reported for digestive gland tissue of bay scallops (*Argopecten irradians*), while another species in this genus (*A. purpuratus)* had much longer telomeres (9.4 kb) (Estabrooks, 2007). Even longer telomere lengths (>20 kb) were recorded for both somatic and germline tissues of sand scallop (*Euvola ziczac*) (Owen *et al.* 2007). Our estimates of telomere length for *S. glomerata* (4-14 kb) fell within this general range. There are no other estimates of telomere lengths in oysters or other bivalve molluscs.

The genome of *S. glomerata* appears likely to contain significant amounts of interstitial or sub-telomeric repeated sequences. Fluorescence *in situ* hybridisation (FISH) or *Bal*31 assay could provide confirmation of this. Interstitial telomeric DNA has also been detected using the TRF assay in the genome of bay scallops (*A. irradians*) (Estabrooks, 2007) but not in the Pacific oyster (*Crassostrea gigas*) (Guo and Allen, 1997) or Portuguese oyster (*C. angulata*) (Cross *et al.* 2005).

The relationship of telomere length with age in *S. glomerata*

There was a general trend for telomere length to decrease with age in the groups of *S. glomerata* sampled in this study although this was only significant at Moreton Bay. At Moreton Bay this decrease was irrespective of tissue or time of sampling and occurred over the younger portion of the lifespan of the species. Individuals in this study ranged in age from two months to four years old which may represent only about a third of the species' life span. Whilst there is no definitive information on the life span of *S. glomerata* in the wild, they are thought to live from six to ten years.

We expected that telomere length would decline with age because of the 'end-replication problem', but other cellular processes associated with ageing can also contribute to telomere attrition. Common features of the ageing process in bivalve species involve a loss of energetic function and higher susceptibility to stress (Philipp and Abele, 2010) and a decline in antioxidant defence and protective chaperones (Ivanina *et al.* 2008). Telomere shortening has been reported to happen irrespective of telomerase activity due to reactive oxygen species (ROS)-related damage (Passos *et al.* 2007). In bivalves periods of shell closure can result in hypoxic or even anoxic conditions for internal tissues, which may in turn affect expression of genes in a variety of physiological pathways and in particular levels of reactive oxygen species (ROS) in their tissues (David *et al*. 2005). Though oysters in this study were only used if their shells were closed we do not have precise information for each cohort on how long the shells were closed between leaving the water and experimentation. It is possible any variation in this length of time may have contributed to some of the observed heterogeneity in observed telomere lengths.

There have been few studies on telomere dynamics with age in other mollusc species, although telomere length was reported to have decreased with age in digestive gland but not heart or adductor muscle of bay scallop *A. irradians* (Estabrooks, 2007). This preliminary study had low replication and as we have found digestive gland DNA is highly susceptible to degradation, this could explain some of the shortening observed by Estabrooks (2007).

The measureable decrease in telomere length with age in *S. glomerata* suggests that telomere maintenance mechanisms which may be active in adult somatic cells oysters are not sufficient to maintain telomere length over the oyster's ten-year life span. In some marine invertebrates such as jellyfish (*Cassiopea andromeda*) (Ojimi and Hidaka, 2010), sea urchins (*Lytechinus variegates* and *Strongylocentrotus franciscanus*) (Francis *et al.* 2006) lobsters (*Homarus americanus*) (Klapper *et al.* 1998b) and sand scallop (*Euvola ziczac*) (Owen *et al.* 2007) ubiquitous telomerase expression has been found to occur in adult somatic and germline tissues and this may provide a mechanism to maintain telomeres (Owen *et al.* 2007). Telomerase activity in *S. glomerata* was not assayed in this study however in future research this would be possible using a procedure such as the telomere repeat amplification protocol (TRAP) assay (Kim *et al.* 1994).

Relationship between telomere length and tissue type

Telomere length tended to be shorter in mantle tissue than in gill tissue particularly in the three and four-year age classes of Moreton Bay oysters. The difference in telomere length between gill and mantle tissue was not significant in Wallis Lake oysters. This was most likely due to the variability between individual oysters within the sample groups and also because telomere length of mantle was only tested across the three and four-year age groups. A larger sample group across a wide age range would be necessary to detect any measurable difference in telomere length.

The decrease in telomere length with age was consistent for mantle and gill tissue. This was contrary to our expectation that the telomere rate of change with age would be higher in gill tissue than in mantle, since the metabolic activity and cell turnover rates associated with maintaining ciliary epithelia in gills would be expected to be greater than that of mantle tissue (Philipp and Abele, 2010). We therefore conclude that the cell turnover rates in the two tissue types is likely to be equal or there is a mechanism of telomere maintenance in gill tissue to compensate for faster cell turnover rates.

Regional effects on the relationship between telomere length and age

Environment may exert a significant effect on the relationship between telomere length and age. It may affect both absolute telomere length and the rate at which it decreases with age. Telomere lengths tended to be shorter in oysters from Wallis Lake than those at Moreton Bay and although telomere lengths decreased with age at both locations, the decrease at Wallis Lake was not significant. Sampling a larger number of oysters over wider age range (up to ten years) would be needed to assess if the decrease in telomere length continued over the entire life span.

The observed differences between locations could be genetically-based since Moreton Bay oysters were derived from commercially produced spat that were part of a breeding program, whereas Wallis Lake oysters were derived from wild caught spat. Hatchery-produced selectively bred spat have been found to grow faster in terms of shell size and whole weight than wild caught spat (Dove and O'Connor, 2009) and it is possible that the two groups also vary in their telomere dynamics over their life cycle.

The disparity between results obtained from Moreton Bay and Wallis Lake oysters may also be a reflection of the different environments in which the oysters have been reared. The location of the Moreton Bay farm is a completely marine environment twenty kilometres from the coast adjacent to the large sand island (Moreton Island). Thus, the area is less subject to the seasonal effects of freshwater runoff that might affect nutrient levels, salinity and turbidity levels of the estuarine environment at Wallis Lake. The two regions have different seasonal water temperatures since Moreton Bay is around seven hundred kilometres north of Wallis Lake on Australia's eastern seaboard. While information regarding environmental effects on ageing of bivalves is limited, studies in other organisms have indicated that the rate of telomere loss was sensitive to cell division rates and environmental circumstances in the cell (Jennings *et al.* 2000; von Zglinicki, 2002).

Temporal effects on telomere length

Our results showed that overall mean telomere lengths of similar aged oysters from a given location can differ by at least 2,000 bp in a two-year interval between sampling times. Validation experiments excluded the possibility that this was due to measurement error. Telomere length decreased with age in 2007 and 2009 sample groups. The decrease in telomere length appeared to occur at a younger age for the 2009 group; however we cannot make this assumption since oysters less than a year old were unavailable in the 2007 group.

Telomere lengths of identically aged oysters, in the one and two-year age classes, were far more uniform in 2009 than any other age groups from Moreton Bay (Figure 3). Observed differences in variation within each age cohort may reflect genetic or environmental effects. Information regarding the husbandry (including culling and selection) of each age cohort in each sample group is incomplete; therefore we cannot assume they faced the same environments during their life history. Even though the 2007 and 2009 Moreton Bay oysters were from the same breeding program, genetic comparisons using IST bands as polymorphic, dominant markers indicate that the oysters within each sample group were more similar to each other than between the 2007 and 2009 groups. It is possible different groups of brood stock are used in the mass selection program over time or different lines may have been supplied to the oyster farmer.

This study on *S. glomerata* was useful for illustrating the heterogeneity of telomere dynamics that can be observed within a given species sampled from their natural setting. Telomere length of *S. glomerata* was generally found to decrease with age for oysters sampled in this research project and it varied amongst tissue types. The relationship between telomere length and age however was weak with individuals of identical age varying significantly in their telomere length. Telomere attrition rates also varied in different regions, and were probably affected by genetic background and temporal effects. Overall, we conclude telomere length is unsuitable as a biomarker of chronological age in oysters. Further research, with greater sample sizes and age classes may tighten the relationship between telomere length and age, however telomere length may prove to be more useful as a molecular indicator of physiological age than a chronological age (Epel *et al.* 2004; Kotrschal *et al.* 2007; Salomons *et al.* 2009). For instance, it would be worthwhile assessing if telomere length could be an indicator of stress, predict future mortality or if the selection of individuals with long telomeres is useful in a breeding program.

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References

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

Bonin, A., Ehrich, D. Manel, S. (2007) Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular

ecologists and evolutionists. *Molecular Ecology* 16, 3737-3758.

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.

Cross, I., Díaz, E., Sánchez, I., Rebordinos, L. (2005) Molecular and cytogenetic characterization of *Crassostrea angulata* chromosomes. *Aquaculture* 247, 135-144.

David, E., Tanguy, A., Pichavant, K., Moraga, D. (2005) Response of the Pacific oyster *Crassostrea gigas* to hypoxia exposure under experimental conditions. *FEBS Journal* 272, 5635-5652.

Dove, M.C., O'Connor, W.A. (2009) Commercial assessment of growth and mortality of fifth-generation Sydney rock oysters *Saccostrea glomerata* (Gould, 1850) selectively bred for faster growth. *Aquaculture Research* 40, 1439-1450.

Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Jason, D.M., Cawthon, R.M. (2004) Accelerated Telomere Shortening in Response to Life Stress. *Proceedings of the National Academy of Sciences of the United States of America* 101, 17312-17315.

Estabrooks, S.L. (1999) The telomeres of the bay scallop, *Argopecten irradians* (Lamarck). *Journal of Shellfish Research* 18, 401-404.

Estabrooks, S.L. (2007) The possible role of telomeres in the short life span of the bay scallop, *Argopecten irradians irradians* (Lamarck 1819). *Journal of Shellfish Research* 26, 307-313.

Francis, N., Gregg, T., Owen, R., Ebert, T., Bodnar, A. (2006) Lack of age-associated telomere shortening in long- and short-lived species of sea urchins. *FEBS Letters* 580, 4713-4717.

Goewert, A.E., Surge, D. (2008) Seasonality and growth patterns using isotope sclerochronology in shells of the Pliocene scallop *Chesapecten madisonius*. *Geo-Marine Letters* 28, 327-338.

Greider, C., Blackburn, E. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* 43, 405-413.

Greider, C., Blackburn, E. (1989) A telomeric sequence in the RNA of *Tetrahymena* telomerase required for telomere repeat synthesis. *Nature* 337, 331-337.

Guo, X.M., Allen, S.K. (1997) Fluorescence in situ hybridization of vertebrate telomere sequence to chromosome ends of the Pacific oyster, *Crassostrea gigas* Thunberg. *Journal of Shellfish Research* 16, 87-89.

Gurney, L.J., Mundy, C., Porteus, M.C. (2005) Determining age and growth of abalone using stable oxygen isotopes: a tool for fisheries management. *Fisheries Research* 72, 353-360.

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.

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.

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. (2003) Calibrating the telomere clock in common terns, *Sterna hirundo*. *Experimental Gerontology* 38, 787-789.

Ivanina, A.V., Sokolova, I.M., Sukhotin, A.A. (2008) Oxidative stress and expression of chaperones in aging mollusks. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 150, 53-61.

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.

Keller, N., Del Piero, D., Longinelli, A. (2002) Isotopic composition, growth rates and biological behaviour of *Chamelea gallina* and *Callista chione* from the Gulf of Trieste (Italy). *Marine Biology* 140, 9-15.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L., Shay, J.W. (1994) Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. *Science* 266, 2011-2015.

Klapper, W., Heidorn, K., Kühne, K., Parwaresch, R., Guido, K. (1998a) Telomerase activity in 'immortal' fish. *FEBS Letters* 434, 409-412.

Klapper, W., Kühne, K., Singh, K.K., Heidorn, K., Parwaresch, R., Krupp, G. (1998b) Longevity of lobsters is linked to ubiquitous telomerase expression. *FEBS Letters* 439, 143-146.

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

Martin, C.S., Bronstein, I. (1994) Imaging of chemiluminescent signals with cooled CCD camera systems. *Journal of Bioluminescence and Chemiluminescence* 9, 145-153.

McShane, P.E., Smith, M.G., Beinssen, K.H.H. (1988) Growth and morphometry in abalone (*Haliotis rubra* Leach) from Victoria. *Marine and Freshwater Research* 39, 161-166.

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

Naylor, J.R., Manighetti, B.M., Neil, H.L., Kim, S.W. (2007) Validated estimation of growth and age in the New Zealand abalone *Haliotis iris* using stable oxygen isotopes. *Marine and Freshwater Research* 58, 354-362.

Nell, J. (2001) The history of oyster farming in Australia. *Marine Fisheries Review* 63, 14-25.

O'Connor, W., Dove, M. (2009) The changing face of oyster culture in New South Wales, Australia. *Journal of Shellfish Research* 28, 803-811.

Ojimi, M., Hidaka, M. (2010) Comparison of telomere length among different life cycle stages of the jellyfish *Cassiopea andromeda*. *Marine Biology* 10.1007/s00227-010-1495-4.

Owen, R., Sarkis, S., Bodnar, A. (2007) Developmental pattern of telomerase expression in the sand scallop, *Euvola ziczac. Invertebrate Biology* 126, 40-45.

Passos, J.F., Saretzki, G., von Zglinicki, T. (2007) DNA damage in telomeres and mitochondria during cellular senescence: is there a connection? *Nucleic Acids Research* 35, 7505-7513.

Payne, R., Murray, D., Harding, S., Baird, D.B., Soutar, D. (2009) *GenStat for Windows (12th Edition) Introduction*. VSN International, Hemel Hempstead.

Philipp, E.E.R., Abele, D. (2010) Masters of Longevity: Lessons from Long-Lived Bivalves – A Mini-Review. *Gerontology* 56, 55-65.

Plohl, M., Prats, E., Martinez-Lage, A., Gonzalez-Tizon, A., Mendez, J., Cornudella, L. (2002) Telomeric localization of the vertebrate-type hexamer repeat, (TTAGGG)(n), in the wedgeshell clam *Donax trunculus* and other marine invertebrate genomes. *Journal of Biological Chemistry* 277, 19839-19846.

Rzhetsky, A., Nei, M. (1992) A simple method for estimating and testing minimum evolution trees. . *Molecular Biology and Evolution* 9, 945-967.

Sakai, M., Okumura, S.I., Yamamori, K. (2005) Telomere analysis of Pacific abalone *Haliotis discus* hannai chromosomes by fluorescence in situ hybridization. *Journal of Shellfish Research* 24, 1149-1151.

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

Sambrook, J., Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schneider, S., Kueffer, J., Roessli, D., Excoffier, L. (2000) *Arlequin: A software for population genetic data analysis*. v2.0. Genetics and Biometry Lab, Department of Anthropology, University of Geneva: Geneva.

Shepherd, S., Al-Wahaibi, D., Al-Azri, A. (1995) Shell growth checks and growth of the Omani abalone *Haliotis mariae*. *Marine and Freshwater Research* 46, 575-582.

Siddeek, M., Johnson, D. (1997) Growth parameter estimates for Omani abalone (*Haliotis mariae*, Wood 1828) using length-frequency data. *Fisheries Research* 31, 169-188.

Szostak, J., Blackburn, E. (1982) Cloning yeast telomeres on linear plasmid vectors. *Cell* 29, 245-255.

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596-1599.

Tarr, R. (1995) Growth and movement of the South African abalone *Haliotis midae*: A reassessment. *Marine and Freshwater Research* 46, 583-590.

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

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, 791-795.

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

von Zglinicki, T., Martin-Ruiz, C.M. (2005) Telomeres as Biomarkers for Ageing and Age-Related Diseases. *Current Molecular Medicine* 5, 197-203.

Watson, J. (1972) Origin of concatemeric T7 DNA. *Nature New Biology* 239, 197-201.

Ying, T.C. (2005) *Evaluation of telomere length as an age-marker in marine species*. Master of Philosophy, Hong Kong.

Table 1: Telomere length estimates (bp) for *Saccostrea glomerata* from Moreton Bay sampled in 2007 and Wallis Lake, aged one to four years using DNA extracted from gill and mantle tissue. Mean values ± standard deviation and number of individuals in parenthesis are presented. Different letters indicate significant differences (P<0.05) between age group means for each tissue type within each location. Coefficient of variation expressed as a percentage (CV%) between sampled animals and between replicate measurements (reps) is included.

Age (y)	Moreton Bay		Wallis Lake	
	Gill	Mantle	Gill	Mantle
1	10580 ± 1543 (6)[a]	10362 ± 812 (6)[a]	-	-
1.5	-	-	8228 ± 1491 (12)[a]	-
3	8477 ± 1620 (6)[b]	7783 ± 1961 (6)[b]	7635 ± 1680 (6)[a]	8369 ± 2031 (6)[a]
4	8829 ± 2884 (6)[ab]	8010 ± 1934 (6)[b]	6783 ± 1454 (5)[a]	7719 ± 1292 (6)[a]
CV% between animals	39	33	35	36
CV% between reps	11	14	11	10

Table 2: Comparison of telomere length estimates for Moreton Bay *Saccostrea glomerata* sampled in 2007 and 2009 based on DNA extracted from gill tissue. Mean values ± standard deviation, and number of individuals in parenthesis are presented. Different letters indicate significant differences (P<0.05) between age groups within each sample year.

Sample Group	Age (y)	Mean telomere length (bp)
2007	1	10580 ± 1543 (6)[a]
	3	8477 ± 1620 (6)[b]
	4	8829 ± 2884 (6)[ab]
2009	0.17	8892 ± 2412 (8)[a]
	1	5918 ± 617 (10)[b]
	2	5672 ± 657 (10)[b]

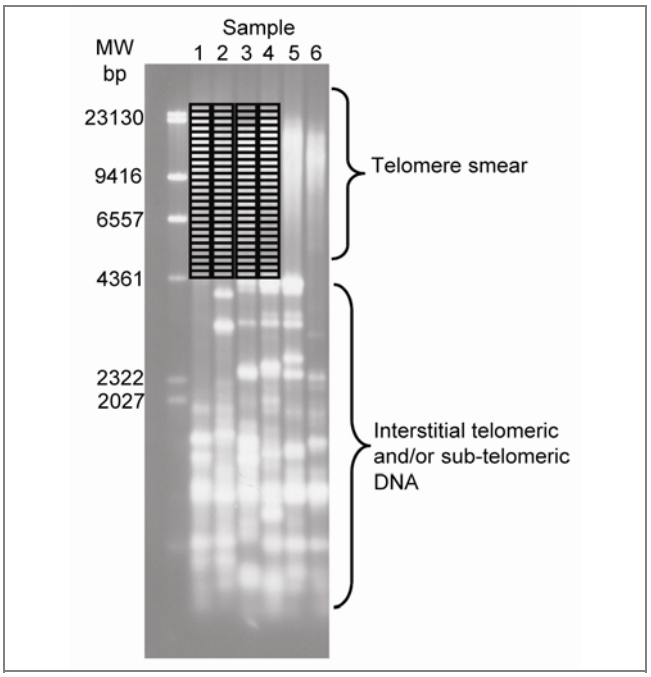

Figure 1: *Saccostrea glomerata* samples from mantle DNA were assayed for mean telomere length. Telomeric DNA appears as a smear above a prominent banding pattern presumed to be derived from interstitial and sub-telomeric repeated DNA. Each lane was overlaid with a grid of 25 boxes before total intensity and molecular weight were estimated for each box.

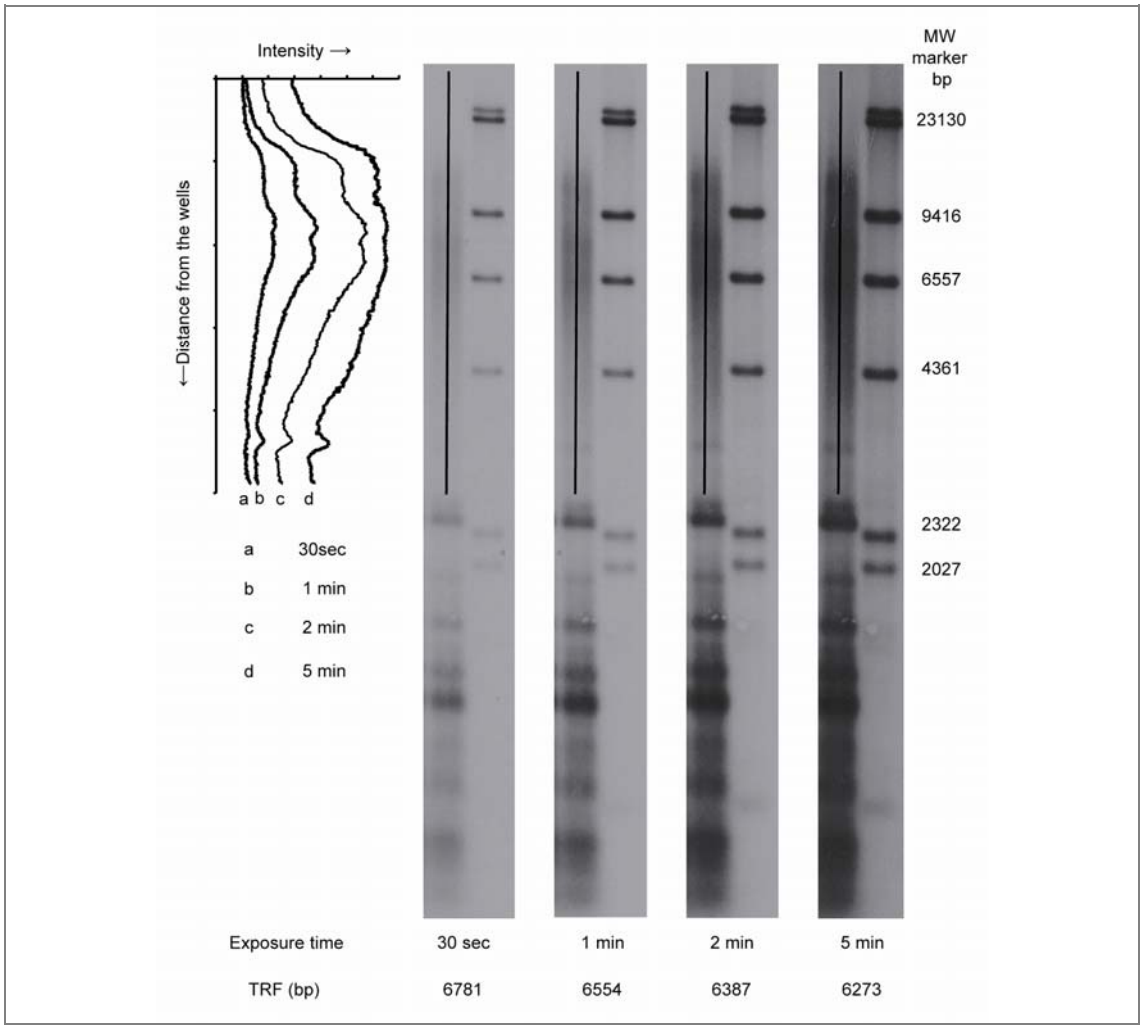

Figure 2: The effect of x-ray saturation on estimates of mean telomere length. The graph on the left shows signal intensity down each lane for the different exposure times. Panels on the right show the effect of increasing exposure time on the intensity of the telomere smear. λHindIII size markers on the right hand side.

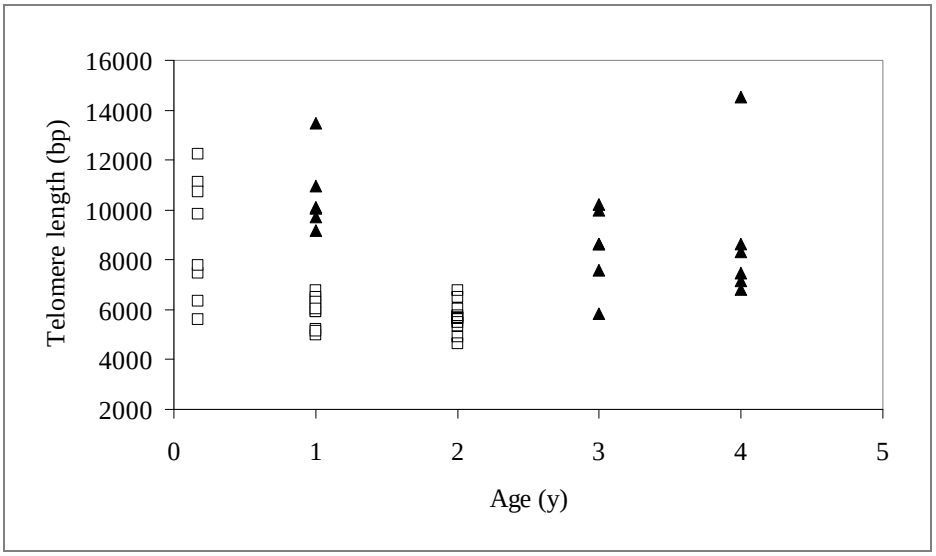

Figure 3 The range of telomere length estimates for *Saccostrea glomerata* individuals in each of three age groups sampled from Moreton Bay in 2007 and 2009. Telomere lengths were based on DNA extracted from gill tissue. Closed symbols represent samples from 2007 and open symbols represent samples from 2009.

Appendix 5.2 – Manuscript in Preparation

Telomere length as a potential biomarker for age in abalone (*Haliotis* spp.)

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Abstract

Age and growth information is crucial to stock assessment models which in turn are central to the sustainable management of abalone fisheries. The range of techniques currently available for collecting age or growth data of abalone are problematic because they are time consuming, laborious, require thorough validation and are often deleterious to the organism. In recent years, a DNA based method using telomere length has been proposed as a potential age biomarker for wild populations of animals. Here we investigate the feasibility of using telomere length to estimate age in abalone. The study included farmed specimens of known age, *Haliotis rubra,* Leach 1814 x *Haliotis laevigata* Donovan, 1808 hybrids and *Haliotis asinina,* Linnaeus, 1758, and wild caught specimens of *Haliotis rubra* where shell size was used as a proxy for age. No relationship was detected between age and telomere length in farmed specimens under four years of age but a strong inverse relationship between shell size and telomere length was detected for wild caught *H. rubra*. The strong relationship between telomere length and shell size suggests that telomere length has the potential to be a useful biomarker of chronological or biological age in *H. rubra* and which could possibly be extended to other abalone and mollusc species.

Keywords

Mollusc, Aging, Age-estimation, DNA, TRF, Australia

Introduction

Abalone (*Haliotis* spp.) belong to a genus of marine gastropods which live in both tropical and temperate waters and comprise an important, high-value fishery throughout the world. *Haliotis rubra*, Leach 1814, and *H. laevigata*, Donovan 1808, are common species in southern Australian waters, together constituting a major portion of the global wild catch. *H. Asinine*, Linnaeus 1758, is a tropical abalone with commercial potential which inhabits Indo-pacific coral reefs

The sustainability of wild fisheries relies upon good management, which includes understanding the age structure of individuals in the population. Age data provides insight into growth rates, reproductive patterns, longevity and mortality and is therefore an integral component of stock assessment models. A variety of techniques have been applied to assess the growth and age of shellfish such as abalone. These include mark-recapture techniques (McShane *et al.* 1988), cohort or length frequency analyses (Shepherd *et al.* 1995; Tarr, 1995; Siddeek and Johnson, 1997), counting growth rings or studying the ratio of isotopes in the carbonate of shells (Gurney *et al.* 2005; Naylor *et al.* 2007; Goewert and Surge, 2008). These techniques have provided useful information in some species but are often laborious, time-consuming, require thorough validation or are deleterious to the organism. The development of a DNA-based technique could offer a quicker, more cost-effective and non-lethal alternative to traditional methods of age-estimation in abalone.

Telomeres are comprised of highly repeated DNA sequences and their associated proteins. They are found at the end of linear eukaryotic chromosomes. Their function is to provide chromosome stability by protecting the ends from degradation and fusion (Blackburn, 1991). Over the lifespan of some animals telomeres have been found to gradually shorten with each mitotic cell division due to a phenomenon known as the 'end-replication problem' (Watson, 1972), but there is additional evidence that telomere attrition rates can be increased by other factors such as oxidative stress (von Zglinicki, 2002). Significant correlations between age and telomere length have been observed for humans and some species of birds (Haussmann and Vleck, 2002; Hausmann *et al*. 2003; Vleck *et al.* 2003), and fish (Nakagawa *et al.* 2004; Ying, 2005; Hatakeyama *et al*. 2008; Hartmann *et al.* 2009). In recent years, telomere length has been suggested as a new age biomarker (von Zglinicki and Martin-Ruiz, 2005), which has particular relevance in natural populations which can be difficult to age using other techniques (Haussmann and Vleck, 2002; Nakagawa *et al.* 2004; Haussmann and Mauck, 2008).

The telomere repeat sequence $TTAGGG_n$ has been determined for several gastropod molluscs, including *Haliotis discus hannai* (Sakai *et al.* 2005), *Haliotis fulgens* and *H. Corrugate* (Gallardo-Escarate *et al.* 2005) as well as the neogastropod *Fasciolaria lignaria* (Vitturi *et al.* 2000), but there have been no studies to date on the use of telomere length as an age biomarker in gastropod species.

The primary aim of this project is to investigate the feasibility of using telomere length to estimate the age of abalone. The study included farmed specimens of known age *H. rubra* x *H. laevigata* hybrids and *H. asinina*, and wild caught specimens *H. rubra* where shell size was used as a proxy for age. We hypothesised there would be an inverse relationship between age and telomere length as has been observed in many species.

Materials and Methods

The relationship between telomere length and age was studied in three species of abalone. Tropical abalone *Haliotis asinina* and hybrid black lip (*H. rubra*) x green lip (*H. laevigata*) abalone of known age were sourced from culture and covered a limited size range and portion of the life history. These samples complemented wild-caught black lip *Haliotis rubra* which overlapped the cultured abalone at smaller shell sizes but extended into larger and presumably older animals.

Sampling and storage of tissues

Farmed black lip x green lip (*Haliotis rubra* x *H. laevigata*) hybrid abalone, aged two, three and four years were obtained from an abalone farm at Dunalley, Tasmania, Australia (42°53'S, 147°48'E). Farmed tropical abalone (*H. asinina*) aged eleven and 18 months were obtained from Bribie Island Research Centre, Queensland, Australia (27°03'S, 153°11'E). Samples of tropical abalone were included to provide a comparison with other species and preliminary data was considered a valuable basis for future work on this species.

Twelve *H. rubra* with shells ranging in size from 140-154 mm, and eighteen with shell sizes ranging from 20-87 mm, were collected in December 2009 and March 2010 respectively from George III Rock Research Area in southern Tasmania, Australia (43°30'S, 146°58'E). Shell size measurements across the longest dimension were made using vernier callipers to the nearest millimetre.

Muscle tissue from the foot was sampled from all abalone specimens except the eleven-months-old *H. asinina*. The latter animals were too small to be dissected and therefore their whole body was removed from the shell and used for telomere length analyses. After sampling, tissues were placed in tubes, immediately snap frozen in liquid nitrogen, and then transferred to a -80°C freezer for long term storage.

Extraction of total genomic DNA and Terminal Restriction Fragment (TRF) assays

The TRF assay provides an estimate of the mean telomere length of all chromosomes in cells of given tissue sample. Total genomic DNA was extracted from frozen tissue using the standard methods of Sambrook and Russell (2001). The resulting DNA was redissolved in TE (Tris-EDTA) buffer pH 8.0, 1 mM Dithiothreitol (DTT) to minimise degradation and oxidation of the telomeric DNA. A range of TRF assays was conducted on abalone genomic DNA from either whole body or muscle tissue. The telomere repeat sequence of abalone was assumed to be TTAGGG, as reported for other gastropods, and this was confirmed by dot blot (data not shown).

The TRF assays were performed using the Telo*TAGGG* Telomere Length Assay kit (Roche Diagnostics Australia Pty. Ltd, Castle Hill, NSW, Australia) with minor modifications. The quantity and integrity of genomic DNA was checked by gel electrophoresis prior to telomere length assays. Genomic DNA (2 µg) was digested with *Hinf*I and *Rsa*I for three hours at 50°C and separated using conventional gel electrophoresis on a 1 x TAE 0.8% agarose gel at 35 V for 20 hours at 4°C.

Samples were loaded in randomised order on every gel to minimise within-gel effects. Each TRF assay was repeated on two and usually three separate occasions. Within-gel variation was estimated by running replicate samples three times within a gel.

DNA was denatured prior to transfer to nylon membrane and subsequent transfer, hybridisation, chemiluminescence and detection were according to the Roche Telo*TAGGG* kit instructions. Chemiluminescent signals from the membrane were captured on x-ray film using multiple exposures as appropriate.

Determination of telomere length

Mean TRF measurements were determined using the Roche Telo*TTAGG* Telomere Length Assay Protocol. X-ray films were scanned using a high definition scanner and images analysed with Quantity One v 4.2.3 imaging software (Biorad, Gladesville, NSW, Australia). Each lane was overlaid with a grid of 25 equal-sized boxes. The total intensity data inside each box and molecular weight at the centre of each box was estimated using Quantity One software. A minimum background subtraction as suggested in the Roche protocol was applied in each lane.

Telomere length was calculated from the TRF assay using the formula

$$\text{Mean TRF} = \Sigma (OD_i)/ \Sigma (OD_i/L_i)$$

where OD is the total chemiluminescent signal in each box and L_i is the length of the TRF fragment at the position 'i'.

Data Analysis

To determine the relationship between size and age data sets generated from farmed *H. rubra* x *H. laevigata* hybrids and *H. asinina* abalone were subjected to analysis of variance (ANOVA) using GenStat (Payne *et al.* 2009). Individual abalones were taken as the independent experimental units, with split-plots adopted for the replicate measurements of the same individuals. Telomere length was the dependent variable, with age group being the independent factor for these analyses. Inspection of residual plots indicated that no transformation of the data was necessary. Least-significance difference (LSD) testing was applied when the ANOVA indicated a significant ($P<0.05$) difference.

The relationship between shell size and telomere length was examined for wild-caught *H. rubra.* Individual abalones were the experimental units. Telomere length was estimated in thirty individuals in three replicate experiments with the arithmetic average of the three experiments being taken as the best estimate for each individual. The degree of the relationship between shell size and telomere length was tested by linear regression.

Results

Farmed *H.asinina* and hybrid *H. rubra* x *H. laevigata* abalone

Telomere lengths of *H.asinina* ranged from 4,465 to 6,668 bp with an average of 5,101 bp. Hybrid abalone (*H. rubra x H. laevigata*) had shorter telomeres than *H.asinina*, with values ranging from 2,478 to 3295 bp and an overall average of 2,994 bp (Table 1). Telomere length did not differ significantly over the measured age range for either species (*H. asinina* ANOVA $F_{1,8} = 3.18$, $P=0.112$; *H. rubra x H. leavigata* $F_{2,12} = 1.65$, $P=0.233$).

For *H.asinina* and hybrid *H. rubra x H. laevigata* abalone, the average coefficient of variation (CV) between replicate experiments was five percent while the coefficient of variation between replicate estimates of a single abalone on the same gel was 1.9%

Telomere length in wild caught *H. rubra*

Telomere lengths of wild caught *H. rubra* ranged from 1,452 bp to 4,390 bp with the overall average being 2,671 bp. The average coefficient of variation between replicate experiments was nine percent. There was a highly significant linear relationship ($r^2 = 0.833$, $P<0.001$) between shell size and mean telomere length in wild caught *H. rubra* (Figure 1). Telomere length decreased from approximately 3,700 bp to 1,500 bp as shell size increased from approximately 40 mm to 150 mm. This represents a decrease in telomere length of more than fifty percent over the sampled size range.

The smaller *H. rubra* (shell sizes <90 mm) tended to be split into two groups. The majority had mean telomere lengths at or below the regression line in Figure 1, while five individuals had mean telomere lengths substantially higher (above regression line in Figure 1). Both groups demonstrated a decline in mean telomere lengths with shell size.

The cultured *H. rubra* x *H. laevigata* abalone had shell sizes that overlapped with the small wild-caught *H. rubra* and their telomere lengths were more similar to the majority group of small *H. rubra.* Shell morphology may vary between species however, in a general comparison, telomere lengths of *H. asinina* (shell sizes 7-42 mm) were longer than the other abalone species

but were more similar to the minority group of five small *H. rubra*.

Discussion

The primary aim of this project was to investigate the feasibility of using telomere length to estimate the age of abalone. Telomere length was successfully measured using TRF assays in three species of abalone *H. asinina, H. rubra* x *H. laevigata* hybrids and *H. rubra* using both farmed and wild caught specimens. There have been no other reports of telomere length studies in gastropod molluscs in the literature. Telomere lengths have been reported for a limited number of bivalves, however. For example, telomere lengths were 0.75-2.3 kb and 9.4 kb for the scallop species *Argopecten irradians* and *A. purpuratus,* respectively (Estabrooks, 2007) and greater than 20 kb for sand scallop (*Euvola ziczac*) (Owen *et al.* 2007). Our estimates of telomere length for abalone (1.4-6.7 kb) fell at the lower end of this general range.

We were unable to detect any relationship between telomere length and age for farmed *H. asinina* and *H. rubra* x *H. laevigata* hybrids, though we expected to observe a decrease in telomere length. This may have been because of the small sample number in each age cohort and that there was only a seven-month and two-year age difference between the youngest and oldest abalone sampled for *H. asinina* and *H. rubra* x *H. laevigata* respectively. The sampled age range represents a small portion of abalone life span which is thought to be approximately fifteen years. It is possible that a significant relationship between telomere length and age would have been observed if greater numbers of individuals over a wider age range had been available for sampling. Further research is therefore necessary to confirm if any relationship between telomere length and age exists in *H. asinina* and *H. rubra* x *H. laevigata* hybrids.

The significant relationship between telomere length and shell size in wild *H. rubra* indicates that telomere length could be used to age abalone as 140-150 mm abalone are expected to be considerably older than the 40-60 mm animals caught at the same reef. Elucidating abalone age would provide a substantial benefit in the assessment of abalone fisheries as reliable estimates of age and growth are still problematic. Future research is required to definitively link age to telomere length and the use of non-lethal method such tagging could facilitate this.

Some individuals in the sampled population have longer telomeres for a given shell size than others indicating there could be some genetic variation in the population for telomere length. There is growing evidence in a range of species that individuals with longer telomeres and lower attrition rates have greater life expectancy than those with short telomeres (Cawthorn, 2002; Pauliny *et al.* 2006; Bize *et al.* 2009; Salomons *et al.* 2009). It is therefore possible that abalone with longer telomeres could have higher fitness or longevity compared to others in the population and this could be of relevance to the aquaculture industry for the selection of brood stock. A longitudinal study of individuals within a population would be needed to demonstrate if longer telomeres or low rates of attrition are predictive of fitness and future life expectancy.

The relationship between shell size and telomere length may indicate that telomere length could be useful as a biomarker of physiological or biological age in abalone. Stress has been found to accelerate telomere attrition in human cells *in vivo* (reviewed by von Zglinicki and Martin-Ruiz, 2005). Additionally, *in vivo* studies with humans (Epel *et al.* 2004) and mice (Kotrschal *et al.* 2007) also found that chronic stress alters telomere dynamics by causing attrition and reducing telomere repair mechanisms. Therefore for a given age, it is possible that *H. rubra* with smaller shells and shorter telomeres may have been exposed to greater cumulative life stress than those with larger shells and longer telomeres. This may be of relevance in monitoring the health of individuals within a population or comparing populations in different areas (environments) where stunting, disease or condition of abalone is in question. This has further relevance to other invertebrate fisheries for example, assessing the condition of commercial scallop beds. Further cross-sectional and longitudinal sampling in different locations would be required to investigate the suitability and scope of using telomere length as a physiological biomarker. In general, telomere length could be a powerful metric to determine the amount of stress experienced by individuals through natural and human perturbation (disease, fishing pressure, climate change).

Conclusion

In summary, telomere length was successfully measured using terminal restriction fragment assays in three species of abalone *Haliotis* asinina, *H. rubra* x *H. laevigata hybrids* and *H. rubra*. Whilst a relationship between telomere length and age was not established in farmed *H. asinina* and *H. rubra* x *H. laevigata* hybrids, the sampled age range was too narrow to test whether a relationship exists. Furthermore, a strong relationship between telomere length and shell size was detected in wild caught *H. rubra.* The use of a quantitative real-time PCR assay such as developed by Cawthorn (2002)

would facilitate future research with greater sample sizes across a greater age range. The strong relationship between telomere length and shell size suggests that telomere length has the potential to be a useful biomarker of chronological or biological age in *H. rubra* and which could possibly be extended to other abalone species.

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Table 1: Mean telomere lengths (n = 5) with standard deviations for *Haliotis asinina* and *Haliotis rubra* x *Haliotis laevigata* hybrids of known age.

H. asinina		*H. rubra* x *H. laevigata*	
Age group	Mean (bp) ±SD	Age group	Mean (bp) ±SD
11 mo	4784 ± 186	2 years	2977 ± 216
18 mo	5266 ± 574	3 years	3119 ± 137
		4 years	2887 ± 243
Grand mean	5025	Grand mean	2994

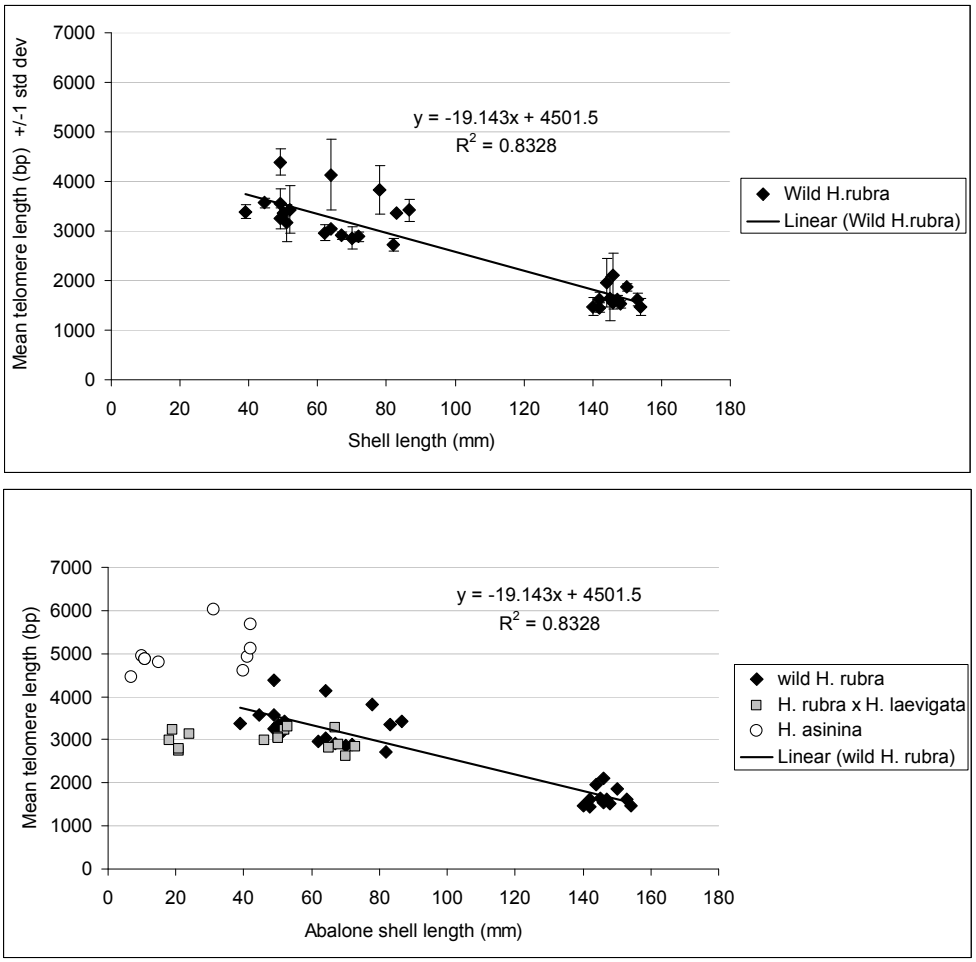

Figure 1: The relationship between telomere length (bp) and shell size (mm) for wild caught *H. rubra* (♦) with corresponding linear regression (upper panel) and *H. asinina* (○) and *H. rubra x H. laevigata* hybrids (■) compared to (♦) *H. rubra* (lower panel).

References

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 B-Biological Sciences* 276, 1679-1683.

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

Cawthorn, R.M. (2002) Telomere measurment by quantitative PCR. *Nucleic Acids Research* 30, 1-6.

Epel, E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Jason, D.M., Cawthon, R.M. (2004) Accelerated Telomere Shortening in Response to Life Stress. *Proceedings of the National Academy of Sciences of the United States of America* 101, 17312-17315.

Estabrooks, S.L. (2007) The possible role of telomeres in the short life span of the bay scallop, *Argopecten irradians irradians* (Lamarck 1819). *J. Shellfish Res.* 26, 307-313.

Gallardo-Escarate, C., Alvarez-Borrego, J., del Rio-Portilla, M.A., von Brand-Skopnik, E., Cross, I., Merlo, A., Rebordinos, L. (2005) Karyotype analysis and chromosomal localization by fish of ribosomal DNA, telomeric (TTAGGG)(N) and (GATA)(N) repeats in *Haliotis fulgens* and *H. corrugata* (Archeogastropoda : Haliotidae). *J. Shellfish Res.* 24, 1153-1159.

Goewert, A.E., Surge, D. (2008) Seasonality and growth patterns using isotope sclerochronology in shells of the Pliocene scallop *Chesapecten madisonius*. *Geo-Marine Letters* 28, 327-338.

Gurney, L.J., Mundy, C., Porteus, M.C. (2005) Determining age and growth of abalone using stable oxygen isotopes: a tool for fisheries management. *Fish Res*. 72, 353-360.

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.

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.

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

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. (2003) Calibrating the telomere clock in common terns, *Sterna hirundo*. *Experimental Gerontology* 38, 787-789.

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

McShane, P.E., Smith, M.G., Beinssen, K.H.H. (1988) Growth and morphometry in abalone (*Haliotis rubra* Leach) from Victoria. *Marine and Freshwater Research* 39, 161-166.

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

Naylor, J.R., Manighetti, B.M., Neil, H.L., Kim, S.W. (2007) Validated estimation of growth and age in the New Zealand abalone *Haliotis iris* using stable oxygen isotopes. *Marine and Freshwater Research* 58, 354-362.

Owen, R., Sarkis, S., Bodnar, A. (2007) Developmental pattern of telomerase expression in the sand scallop, *Euvola ziczac. Invertebrate Biology* 126, 40-45.

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

Payne, R., Murray, D., Harding, S., DB, B., Soutar, D. (2009) *GenStat for Windows (12th Edition) Introduction*. VSN International, Hemel Hempstead.

Sakai, M., Okumura, S.I., Yamamori, K. (2005) Telomere analysis of Pacific abalone *Haliotis discus* hannai chromosomes by fluorescence in situ hybridization. *J. Shellfish Res*. 24, 1149-1151.

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

Sambrook, J., Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Shepherd, S., Al-Wahaibi, D., Al-Azri, A. (1995) Shell growth checks and growth of the Omani abalone *Haliotis mariae*. *Marine and Freshwater Research* 46, 575-582.

Siddeek, M., Johnson, D. (1997) Growth parameter estimates for Omani abalone (*Haliotis mariae*, Wood 1828) using length-frequency data. *Fish Res.* 31, 169-188.

Tarr, R. (1995) Growth and movement of the South African abalone *Haliotis midae*: A reassessment. *Marine and Freshwater Research* 46, 583-590.

Vitturi, R., Colomba, M.S., Gianguzza, P., Pirrone, A.M. (2000) Chromosomal location of ribosomal DNA (rDNA), (GATA)(n) and (TTAGGG)(n) telomeric repeats in the neogastropod *Fasciolaria lignaria* (Mollusca : Prosobranchia). *Genetica* 108, 253-257.

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: 791-795.

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

von Zglinicki, T., Martin-Ruiz, C.M. (2005) Telomeres as Biomarkers for Ageing and Age-Related Diseases. *Current Molecular Medicine* 5, 197-203.

Watson, J. (1972) Origin of concatemeric T7 DNA. *Nature New Biology* 239, 197-201.

Ying, T.C. (2005) *Evaluation of telomere length as an age-marker in marine species*. Master of Philosophy thesis, University of Hong Kong.

Appendix 5.3 – Submitted Manuscript

Telomere length analysis in crustacean species: *Metapenaeus macleayi*, *Sagmariasus verreauxi*, *Jasus edwardsii* and *Ranina ranina*

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Abstract

Age and growth estimates in crustaceans have been historically problematic and present significant challenges to researchers. Current aging techniques provide valuable data but also suffer from disadvantages. Telomeric DNA has been proposed as an age-biomarker because it has been observed to shorten with age in some species. In this study the feasibility of using telomere length (TL) to estimate age was examined in school prawns, *Metapenaeus macleayi,* spiny lobsters *Sagmariasus verreauxi* and *Jasus edwardsii* and spanner crab *Ranina ranina*. Carapace length was used as a surrogate for age and terminal restriction fragment assays were used to test the relationship between TL and size. Degradation of telomeric DNA with time during storage significantly influenced TL estimates, particularly for *M. macleayi*. Telomere lengths obtained from species in this study were 10-20 kb. No relationship between carapace length and TL was detected for any of the test species and TL did not differ between male and female *M. macleayi.* Telomere lengths of *J. edwardsii* pueruli were unexpectedly shorter than *J. edwardsii* adults. The suitability of TL as an age biomarker in crustaceans may be limited but further research is recommended to elucidate telomere dynamics in these species with different life histories and lifespans.

Keywords

Age biomarker, age estimation, Australia, crustacean, telomere length, TRF

Introduction

Accurate assessments of age are required for understanding growth, maturity, reproduction, longevity and mortality of individuals in a wild population (Campana, 2001). These are fundamental considerations in stock assessment models for the sustainable management of fisheries.

Age and growth estimates in crustaceans have been historically problematic and therefore present significant challenges for researchers. Commonly used indirect methods include tag-recapture studies or size-frequency distributions and although providing valuable data, these can be inaccurate, subject to biases, and deleterious to the animal (reviewed by Wahle and Fogarty, 2006). Commonly used direct methods include measurements of anatomical characteristics such as size and weight which are only weakly correlated with age in crustaceans (Zheng *et al*. 1995; Uglem *et al.* 2005). In the last ten years or so, many researchers have also relied upon the accumulation of lipofuscins, pigments which accumulate with age in neural tissue, to estimate the age of crustaceans (Sheehy *et al.* 1994; Ju *et al.* 1999; Uglem *et al.* 2005; Sheehy, 2008). Success using lipofuscin pigments has been mixed and accuracy of the method is still being debated

(Harvey *et al.* 2008; Sheehy, 2008). Lipofuscin accumulation is not independent of environmental effects, particularly temperature, the method is costly and time consuming and it is destructive to the animal (Wahle and Fogarty, 2006). Therefore the development of an alternative method which is low-cost and non-lethal to reliably estimate age in crustaceans would be a key improvement for fisheries management.

Telomeres are structures at the ends of linear chromosomes which are composed of highly repeated DNA sequences and their associated proteins. Their function is to protect the ends of the chromosomes from degradation and fusion (Blackburn, 1991). In many vertebrate somatic cells, telomeres have been found to shorten with each mitotic cell division over the animal's life span owing to incomplete replication of the DNA (Watson, 1972). This shortening has been found to be correlated with age in mammals, including humans and some species of birds (Haussmann and Vleck, 2002; Haussmann *et al.* 2003; Vleck *et al.* 2003; Nakagawa *et al.* 2004) and fish (Hatakeyama *et al.* 2008; Hartmann *et al.* 2009). It has also been shown to be correlated with age in oysters and shell size in abalone (Godwin *et al*., unpublished). Therefore telomere length has been suggested as a possible age-biomarker in animals which are difficult to age using conventional methods (von Zglinicki and Martin-Ruiz, 2005; Haussmann and Mauck, 2008). The telomere repeat sequence of crustaceans has been reported to be $(TTAGG)_n$ which is common in many arthropod species (Klapper *et al.* 1998; Sahara *et al.* 1999; Lang *et al.* 2004; Vítková *et al.* 2005; Elmore *et al*. 2008). Although the telomere repeat sequence of a number of crustaceans has been identified there have not yet been any detailed studies of telomere length in crustaceans.

In this study we examine the relationship between telomere length and age (as indicated by size) in four crustacean species with different life spans and life characteristics. Test species included school prawns, *Metapenaeus macleayi* (Haswell, 1879), two species of spiny lobsters *Sagmariasus verreauxi* (H. Milne Edwards, 1851) formerly *Jasus verreauxi*, and *Jasus edwardsii* (Hutton, 1875) and spanner crab *Ranina ranina* (Linnaeus, 1758). These crustaceans are commercially important species and are common in Australian waters. School prawns are a short lived species (lifespan 12-18 months) (Montgomery *et al.* 2010) which are endemic to estuarine and in-shore ocean waters along the east coast of Australia from southern Queensland to eastern Victoria. Juvenile and sub-adult prawns inhabit estuaries, generally near seagrass beds while adults are predominantly found in ocean waters, but may occur in estuaries (Coles and Greenwood, 1983; Ruello, 1977).

Sagmariasus verreauxi and *J. edwardsii* have relatively long life spans, approximately 30 years for *S. verreauxi* (Montgomery *et al*. 2009) and potentially longer for *J. edwardsii*. *Sagmariasus verreauxi* occurs along the east coast of Australia and North Island of New Zealand and is distributed in warmer waters (Montgomery *et al.* 2009). *Jasus edwardsii* is widely distributed around southern mainland Australia, Tasmania, and New Zealand (Booth, 2007). The life span of *Ranina ranina* is still not known but may be between 7-15 years. They are commonly found offshore in subtropical and warmer temperate waters on the east coast of Australia (Brown, 1986; Kirkwood *et al.* 2005).

Lobsters such as *S. verreauxi* and *J. edwardsii* exhibit indeterminate growth patterns (Wahle and Fogarty, 2006) and despite their shorter lifespans, *Ranina ranina* (I. Brown, pers comm.) and *M. macleayi* are also likely to exhibit indeterminate growth. Thus these species grow continuously throughout their life-span and theoretically have no upper size limit.

The aim of this study was to determine whether telomere length may be a suitable age biomarker in crustaceans. If suitable, a molecular aging method which is based on telomere length could be developed thereby allowing age to be estimated from small tissue samples. This may provide a non-lethal and low cost alternative to conventional methods of collecting age data. Our strategy was to compare telomere lengths in at least two divergent age/size cohorts per species and add data from other cohorts if any differences were indicated. Telomere length can be estimated from very small quantities of tissue using PCR methods (Cawthorn, 2002) however, in the absence of sequence data for these test species, the Terminal Restriction Fragment (TRF) assay was used in this initial study. We hypothesised that there would be an inverse relationship between telomere length and age as has been observed in many vertebrate species.

Materials and Methods

Sampling and storage of tissues

Size (as indicated by carapace length) was used as a surrogate for age since wild specimens of unknown age were used for each species. Three broad size classes of *M. maclayei* prawns comprising approximately equal numbers of male and females were sampled for telomere length analyses. Small and medium sized prawns were obtained from Lake Wooloweyah, an estuarine lake near Yamba, New South Wales. Large

M. maclayei were also caught from the ocean adjacent to Yamba. Carapace length was recorded for each individual before abdominal tissue from the tail area was removed from the exoskeleton for later analyses (Table 1).

Sagmariasus verreauxi encompassing four general size classes from pueruli to very large (carapace lengths 11-159 mm) were used in the study (Table 1). Pueruli and three-year-old individuals were obtained from the Cronulla Fisheries Research Centre, New South Wales. The three-year-old lobsters were caught in waters off Port Hacking as recently-settled pueruli and small lobsters had been grown in captivity for approximately three years at the time of sampling. Large and very large lobsters were caught in waters off Coffs Harbour, New South Wales (Table 1).

Adult *J. edwarsdsii* (carapace lengths 80-120 mm) were caught from the Sandstone Bluff area, Tasmania and pueruli were caught in eastern Tasmanian waters (Table 1). *Ranina ranina* crabs of varying size (carapace lengths 70-140 mm) were caught offshore from the wild under permit by a commercial fisher near Caloundra, Queensland (Table 1).

Lobsters and crabs were transported to the laboratory alive and euthanased in ice slurry. After sampling, all tissues (Table 1) were placed in tubes, immediately snap frozen in liquid nitrogen, and then transferred to a -80°C freezer for long term storage.

Extraction of total genomic DNA and TRF assays

The TRF assay was used to estimate the mean telomere length in cells of given tissue samples. High molecular weight genomic DNA was extracted from frozen tissue using standard methods (Sambrook and Russell, 2001). Extracted crustacean DNA appeared to be susceptible to degradation during storage, and therefore to minimise the co-purification of contaminating material, a smaller quantity of fresh tissue was used during the cell lysis step. As such, 50 mg fresh tissue was digested per millilitre of digestion buffer (10mM Tris pH 8.0, 5.0 mM EDTA pH 8.0, 0.5% SDS, 200µg ml^{-1} Proteinase K). Proteins and other contaminants were removed by salt precipitation in 5 M potassium acetate and phenol/chloroform extractions.

Different combinations of temperatures and incubation periods were found to be optimal for cell lysis and digestion of fresh tissue for each species (Table 2). The resulting DNA was redissolved in TE (Tris-EDTA) buffer pH 8.0, with 1 mM Dithiothreitol (DTT) added to minimise degradation and oxidation of the telomeric DNA**.** All DNA samples were stored at 4°C, rather than -20°C, to minimise shearing during the freeze thaw process. The quality and quantity of genomic DNA was assessed by gel electrophoresis prior to each assay. Quality was assessed as being high if the DNA had a high molecular weight band migrating more slowly than the 23 kb molecular weight marker and there was minimal presence of low molecular weight fragments (Figure 1). Degradation was evidenced by the majority of DNA appearing as a very low molecular weight band or smear. Any apparently degraded DNA samples were discarded prior to TRF assay and exclusion of obviously degraded samples was a standard part of the experimental design.

Terminal restriction fragment assays were conducted on crustacean genomic DNA from either abdominal tissue minus the exoskeleton, muscle or gill tissue. The TRF assays were performed using the Telo*TAGGG* Telomere Length Assay kit (Roche Diagnostics Australia Pty. Ltd, Castle Hill, NSW, Australia) with minor modifications (Table 2).

Although complete digestion of *M. macleayi* genomic DNA was obtained using restriction endonucleases *Hin*fI and *Rsa*I from the Roche kit, preliminary experiments revealed only partial digestion was achieved for *S. verreauxi*, *J. edwardsii* and *R. ranina* DNA. Therefore, a range of other frequently-cutting endonucleases such as *Aci*I, *Alu*I *, Hpy*CH4V, *Mse*I, *Taq*I, *Tsp*5091 were tested in various combinations on genomic DNA from *S. verreauxi*, *J. edwardsii* and *R. ranina*. The most efficient digestion for all three species was obtained using a combination of *Alu*I and *Hpy*CH4V. Two micrograms of genomic DNA per sample was digested with the appropriate enzyme combination for three to five hours at 50°C.

Telomere smears of *S. verreauxi* and *R. ranina* were not efficiently resolved using conventional agarose gel electrophoresis so fluctuating inverted-field gel electrophoresis (FIGE) (Biorad, Australia) was employed. A number of FIGE programs were tested that were designed to separate DNA fragments ranging from 25-150 kb, 25-75 kb and 25-50 kb. The 25-50 kb program was found to be the best for separating and spreading the telomeric fragments from *S. verreauxi* and *R. ranina*. A higher range molecular weight DNA size marker was used (λ Monocut mix, plus λ HindIII molecular weight marker) (New England BioLabs Inc., USA) which allowed size estimation of DNA fragments up to 50 kb.

DNA was denatured prior to transfer to a nylon membrane and subsequent transfer, hybridisation, chemiluminescence and detection were according

to the Roche Telo*TAGGG* kit instructions except that a 3' Digoxigenin (DIG) end-labelled $(TTAGG)_6$ probe (Sigma Genosys Pty. Ltd., Castle Hill, NSW, Australia) was used in place of the probe supplied with the kit. TRF assays were conducted in triplicate with samples loaded on each gel in randomised order.

Determination of telomere length

Mean TRF measurements were determined using the Roche Telo*TTAGG* Telomere Length Assay Protocol with images captured on x-ray films. Each lane was overlaid with a grid of 25 equal-sized boxes. The total intensity data inside in each box and molecular weight at the centre of each box was estimated using Quantity One software. A minimum background subtraction as suggested in the Roche protocol was applied in each lane and telomere length was estimated using the formula:

$$\text{Mean TRF} = \Sigma (OD_i)/ \Sigma (OD_i/L_i)$$

where OD_i is the total chemiluminescent signal in each box and L_i is the length of the TRF fragment at the position 'i'.

Data analysis

School prawns (*Metapenaeus macleayi*)

The main effects of size (as measured by carapace length) and sex on telomere length were investigated in *M. macleayi* using equal numbers of male and female prawns from each broad size category, small, medium and large. The effect of DNA degradation during storage time on telomere length was also investigated in the data analysis. GenStat (Payne *et al.* 2009) was used to fit general linear models (McCullagh and Nelder, 1989) for telomere length. DNA storage time and carapace lengths were continuous variates, and sex was a factor. Residual plots indicated the Normal model with identity link was acceptable. Splines and polynomials of the variates were considered, with the latter (with degree two) adopted for interpretation. All interactions were trialled, but dropped from the final model as they were not significant (P>0.05).

Lobsters (*Sagmariasus verreauxi* and *Jasus edwardsii*)

General linear models were also fitted to data sets generated for each lobster species using GenStat (Payne *et al.* 2009). Individual animals were taken as the independent experimental units, with split-plots adopted for the replicate measurements of the same individuals. Telomere length was the dependent variable, with size or carapace length being the independent factor for these analyses. Inspection of residual plots indicated that no transformation of the data was necessary. Post-hoc unpaired t-tests were used to compare data which could not be included in the same general linear model due to aliasing.

Spanner crabs (*Ranina ranina*)

Data analyses were not required for *R.ranina* since meaningful data were not obtained using the experimental conditions tested in this study.

Results

School prawns (*Metapenaeus macleayi*)

There was no significant difference between female and male prawns in their telomere lengths ($F_{1,176}$ = 1.49; P=0.224). Mean telomere lengths of 10020 ± SE 250 bp and 9709 ± SE 215 bp were recorded for female and males, respectively. The relationship between carapace length and telomere length for both sexes was also non-significant ($F_{2,176}$ = 1.59; P=0.207).

Degradation of telomeric DNA relative to storage time had a significant effect on telomere length measurements in prawns. Results from the general linear models showed telomere length values decreased significantly as duration of DNA storage increased ($F_{2,176}$ = 35.55; P<0.001); Figure 2). Adjusted values shown in the graph are observed values adjusted for other terms in the model. Therefore if *M. macleayi* DNA is stored for a number of weeks before TRF assays are conducted, observed telomere lengths can be around thirty percent lower than they might have been if the assays had been conducted within seven days of extraction. This reduction in telomere length occurred despite validating the quality of genomic DNA by gel electrophoresis immediately prior to the TRF assay. Genomic DNA was only used if it had remained as a high molecular weight band such as that shown in Figure 1.

Lobsters (*Sagmariasus verreauxi*)

Telomere length means of *S. verreauxi* were close to 16-18 kb, approximately twice the length of that recorded for *M. macleayi*. There was little variation in telomere lengths between the sampled tissues of *S. verreauxi* (Table 3). Amongst adult lobsters there was no difference in telomere lengths between gill and muscle tissue ($F_{1,94}$ = 2.89; P=0.092) . The mean telomere length of puerulus abdominal tissue was 17908 ± SE 366 bp which was also not significantly different from the combined mean of 16961 ± SE 1131 bp for gill and muscle tissue of older lobsters (t = 0.80; P=0.43).

No relationship between size and telomere length was detected amongst the *S. verreauxi* in this study. The effect of size on telomere length was only tested amongst adult lobsters. Data obtained from puerulus were excluded since they were based on a mixture of abdominal tissues whereas telomere lengths of older lobsters were based on single tissues, either gill or muscle tissue. Amongst the remaining three size classes (three years old, large and very large lobsters) there was no significant difference in telomere length ($F_{2,93}$ = 2.14; P=0.124) (Table 3). Furthermore, when carapace length was used as a more precise measure of size and the relationship between telomere length and carapace length tested by linear regression the result was also non-significant ($F_{1,94}$ = 0.99; P=0.323). The coefficient of variation (CV) in telomere length between sampled puerulus individuals was 11.6% and 21.2% amongst adult individuals.

Like *M. macleayi*, the duration of DNA storage time had a significant effect on telomere length estimates of *S. verreauxi* ($F_{1,94}$ = 9.11; P=0.003) (Figure 3). Several *S. verreauxi* DNA extracts had been stored for nearly ten months before assay and although the quality of the DNA was checked immediately prior to assay, there was an average decline in telomere length estimate of 24.8% over three hundred days. The degradation observed for *S. verreauxi* occurred more slowly than that observed for *M. macleayi* where a similar degree of degradation occurred in one tenth of the time.

Lobsters (*Jasus edwardsii*)

Telomere length assays were successfully conducted on *J. edwardsii* individuals using DNA extracted from abdominal tissue of nine pueruli and muscle tissue of nine adults. Gill tissue was unavailable for assaying. Two of the pueruli samples degraded during the assay and therefore results are based on seven pueruli and nine adults. The average telomere length of pueruli was 10,259 bp ± SE 139 which was significantly shorter than for adult *J.edwardsii* with 15,124 bp ± SE164 (t = 22.6; P<0.001). These telomere lengths are intermediate between the average telomere lengths recorded for *M. macleayi* and *S. verreauxi*. The apparent lengthening of *J.edwarsii* telomeres as the animals get larger (older) is by no means conclusive since the results are confounded by the different tissues used for each group of animals. The observed result may be solely an effect of the presence of non-muscle tissues in pueruli samples.

In adult *J.edwardsii* the relationship between carapace length and telomere length was non-significant ($F_{1,7}$ = 0.37; P=0.563). A CV of 10% was recorded amongst individual pueruli and 4.8% amongst adult individuals for telomere length. While the variation in telomere lengths amongst pueruli of *J. edwardsii* and *S. verreauxi* was comparable, the variation amongst *S. verreauxi* adults was approximately four times greater than that amongst *J. edwardsii* adults. This might reflect greater variability in telomere length in the *S. verreauxi* population but could be an artefact caused by the degradation of some of the *S. verrreauxi* samples.

The number of days which DNA had been stored before TRF assay did not significantly affect telomere length estimates of *J. edwardsii* ($F_{1,17}$ = 0.03;P=0.868). Susceptibility to *J. edwardsii* DNA to degradation cannot however be ruled out because the difference between the shortest and longest amount of time that elapsed between DNA extraction and TRF assay was only ten days (18 days vs. 28 days).

Spanner crab (*Ranina ranina*)

It was not possible to obtain estimates of *Ranina ranina* telomere lengths. Complete digestion of *R. ranina* genomic DNA was not achieved for any of the tested combinations of enzymes despite measures employed to optimise the purity of extracted DNA. During electrophoresis with either conventional apparatus or FIGE, a significant portion of the DNA remained in the well or smeared down from the well to above the 20 kb molecular weight marker. Further work would be required to optimise experimental conditions before reliable estimates of telomere length can be made in this species.

Discussion

Methodological considerations

Results from this study showed that telomeric DNA of crustaceans is particularly susceptible to degradation during extraction and upon storage affecting the accuracy of telomere length estimations. This was particularly important for *M. macleayi* where telomere length estimates were up to thirty percent lower if DNA had been stored for a few weeks. The tissues used for *M. macleayi* DNA extractions were mainly muscle tissue from the abdomen, however other tissues associated with the digestive tract could also have been present since these were not dissected away. This may have contributed to the problems of degradation since in previous studies we have found that DNA from digestive tissue of other marine organisms such as oysters is very susceptible to degradation (data not shown). Takubo and others (2002), in working with DNA from a wide range of human tissues, also highlighted the need to check for DNA degradation by autolysis and artificial changes. They checked DNA samples prior to TRF assay using pulsed field

electrophoresis and found that a considerable number samples had to be omitted from their study.

Methodological measures undertaken to reduce degradation of telomeric DNA minimised but never completely eliminated the problem. These measures included optimising extraction conditions and including a reducing agent in storage buffer to prevent oxidative damage. Degradation of telomeric DNA during storage was not necessarily apparent before TRF assay since within-gel visualisation of intact genomic DNA prior to assay did not show a noticeable reduction in quality with time. This suggests that telomeric DNA may be degrading at a faster rate than other genomic DNA and therefore for accurate estimates of telomere length, assays should be conducted as soon as possible after the DNA has been extracted from the tissue. The effect of storage time on the integrity of telomeric DNA is an important consideration for telomere researchers and has the potential to contribute to the variability of published data. Experiments conducted on a range of samples over a controlled time period would be necessary to fully quantify the susceptibility of stored telomeric DNA to degradation and should be a necessary first step to estimating telomere lengths from crustaceans.

The relationship between telomere length and age

The telomere lengths of the three crustacean species in this study ranged from 10-20 kb and were not dissimilar to telomere lengths reported for other aquatic species spanning a variety of taxa (Elmore *et al.* 2008). Although the telomere repeat sequence has been identified in a number of crustaceans including lobster *Homarus americanus* (Klapper *et al.* 1998), green sea crab *Carcinus maenus* (Elmore *et al.* 2008), and prawns *Penaeus japonicus* (Lang *et al.* 2004) and *P. semisulcatus* (Okazaki *et al.* 1993) there have not been any detailed studies of telomere length in crustaceans.

There was no relationship between telomere length and carapace length for the three crustacean species included in this study despite their different life spans. It was not surprising for the two lobster species since telomerase, the enzyme involved in telomere repair and maintenance mechanisms, has been found to be highly active in all fully differentiated tissues of the American clawed lobster *Homarus americanus* (Klapper *et al.* 1998). Lobsters are animals with high regenerative potential that exhibit indeterminate growth, negligible senescence and have a low rate of tumour formation and it has been suggested that sustained telomerase expression is necessary to facilitate this continued growth and replacement of tissues across their lifespan (Gomes *et al.* 2010: Klapper *et al.* 1998; Vogt, 2008). The lack of apparent shortening of telomeres between very young (puerulus) and very large adults suggests that telomere maintenance mechanisms are functioning across the life span of *S. verreauxi* and *J. edwardsii*. Future research using a technique such as the TRAP assay (Kim *et al.* 1994) would provide information regarding the relevance of telomerase expression to telomere maintenance in these crustaceans. Furthermore, it is possible that additional telomere maintenance mechanisms are active in spiny lobsters since telomeres were significantly longer in *J. edwardsii* adults than in pueruli. Further research would be necessary to elucidate these mechanisms.

Genetic and environmental effects may have contributed to the variability in telomere lengths amongst individuals within each lobster species. Each group of individuals was sourced from different places and in some cases the exact location of sampling was not known (Table 1). The large variability in the sampled groups may therefore have limited our ability to detect an association between telomere length and age. Larger sample sizes from more controlled sample groups would therefore be desirable for a more detailed analysis of telomere dynamics and age in spiny lobsters.

We expected that telomere attrition with age may have been observed in a short-lived crustacean such as *M. macleayi*. This prawn species has a short life span of about 18 months. However this was not the case. The age of the wild caught *M. macleayi* was unknown and although size is a crude surrogate for age we can predict from the life cycle of *M. macleayi* that small individuals caught from the estuarine lake would have been younger than those from the ocean. This is because juveniles inhabit estuaries, whereas adults are predominantly found in ocean waters (Coles and Greenwood, 1983; Ruello, 1977).

The absence of telomere shortening with age in *M. macleayi* suggests that telomere maintenance mechanisms are present in short lived as well as long lived crustaceans. Additionally these maintenance mechanisms do not seem to vary between sexes of *M. macleayi*. High telomerase expression has been detected in all differentiated tissues of green sea crab *Carcinus maenus* which has a life span of five to six years (Elmore *et al.* 2008) and both reproductive and somatic tissues of *Penaeus japonicus* (Lang *et al.* 2004). Elmore and others (2008) found telomerase activity at significant levels in a variety of tissues of both long and short lived aquatic species of diverse taxa. Furthermore they suggested that the high levels of

telomerease expression in tissues of aquatic animals are not related to longevity but to their ability to regenerate tissues after injury. Not all crustaceans have the ability to regenerate limbs, therefore further research to compare telomere dynamics between crustacean species that vary in this ability may assist scientists to increase their understanding of the key cellular processes involved in damage and repair mechanisms.

For all the species examined in this study there was no significant difference in mean telomere length between the size classes. However, larval and juvenile life history stages of these organisms were not examined. This is a period of significant transition and may represent the greatest growth period in the life history of a crustacean and may also be the period of the most dramatic change in telomere length. Differences in telomere lengths have been found in different life history stages of the jelly fish *Cassiopea Andromeda*, whereby the bell region of the medusa exhibited significantly longer telomeres than polyps (Ojimi and Hidaka, 2010). In future work comparisons between early larval, metamorphic, juvenile and adult life stages may help elucidate mechanisms of crustacean telomere dynamics.

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References

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

Booth, J. D. (2007) Jasus Species. In: *Lobsters: Biology, Management, Aquaculture and Fisheries*, pp. 340-358, Blackwell Publishing Ltd.

Brown, I. W. (1986) Population biology of the spanner crab (*Ranina ranina*) in south-east Queensland. Final Project Report, Southern Fisheries Centre, Department of Primary Industries and Fisheries, Queensland (106pp.).

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.

Cawthorn, R. M. (2002) Telomere measurment by quantitative PCR. *Nucleic Acids Research* 30, 1-6.

Coles, R., and Greenwood, J. (1983) Seasonal movement and size distribution of three commercially important Australian prawn species (Crustacea : Penaeidae) within an estuarine system. *Marine and Freshwater Research* 34, 727-743.

Elmore, L. W., Norris, M. W., Sircar, S., Bright, A. T., McChesney, P. A., Winn, R. N., and Holt, S. E. (2008) Upregulation of Telomerase Function During Tissue Regeneration. *Exp. Biol. Med.* 233, 958-967.

Gomes, N. M. V., Shay, J. W., and Wright, W. E. (2010) Telomere biology in Metazoa. *FEBS Letters* 584, 3741-3751.

Hartmann, N., Reichwald, K., Lechel, A., Graf, M., Kirschner, J., Dorn, A., Terzibasi, E., Wellner, J., Platzer, M., Rudolph, K. L., Cellerino, A. and 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.

Harvey, H. R., Secor, D. H., and Ju, S. J. (2008) The use of extractable lipofuscin for age determination of crustaceans: Reply to Sheehy (2008). *Marine Ecology-Progress Series* 353, 307-311.

Hatakeyama, H., Nakamura, K.-I., Izumiyama-Shimomura, N., Ishii, A., Tsuchida, S., Takubo, K., and 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.

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

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

Haussmann, M. F., Vleck, C. M., and Nisbet, I. C. T. (2003) Calibrating the telomere clock in common terns, *Sterna hirundo*. *Experimental Gerontology* 38, 787-789.

Ju, S.-J., Secor, D. H., and Harvey, H. R. (1999) Use of extractable lipofuscin for age determination of blue crab *Callinectes sapidus*. *Marine Ecology Progress Series* 185, 171-179.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L. C., Coviello, G. M., Wright, W. E., Weinrich, S. L. and Shay, J. W. (1994) Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. *Science* 266, 2011-2015.

Kirkwood, J. M., Brown, I. W., Gaddes, S. W., and Hoyle, S. (2005) Juvenile length-at-age data reveal that spanner crabs (*Ranina ranina*) grow slowly. *Marine Biology* 147, 331-339.

Klapper, W., Kühne, K., Singh, K. K., Heidorn, K., Parwaresch, R., and Krupp, G. (1998) Longevity of lobsters is linked to ubiquitous telomerase expression. *FEBS Letters* 439, 143-146.

Lang, G.-h., Wang, Y., Nomura, N., and Matsumura, M. (2004) Detection of Telomerase Activity in Tissues and Primary Cultured Lymphoid Cells of *Penaeus japonicus*. *Marine Biotechnology* 6, 347-354.

McCullagh, P., and Nelder, J. A. (1989) *Generalized Linear Models* (2nd Edition). Chapman and Hall, London.

Montgomery, S. S., Liggins, G. W., Craig, J. R., and McLeod, J. R. (2009) Growth of the spiny lobster *Jasus verreauxi* (Decapoda: Palinuridae) off the east coast of Australia. New Zealand *Journal of Marine and Freshwater Research* 43, 113-123.

Montgomery, S. S., Walsh, C. T., Haddon, M., Kesby, C. L., and Johnson, D. D. (2010) Using length data in the Schnute Model to describe growth in a metapenaeid from waters off Australia. *Marine and Freshwater Research* 61, 1435-1445.

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

Ojimi, M., and Hidaka, M. (2010) Comparison of telomere length among different life cycle stages of the jellyfish *Cassiopea andromeda*. *Marine Biology*, 10.1007/s00227-010-1495-4.

Okazaki, S., Tsuchida, K., Maekawa, H., Ishikawa, H., and Fujiwara, H. (1993) Identification of a pentanucleotide telomeric sequence, (TTAGG)n, in the silkworm *Bombyx mori* and in other insects. *Mol. Cell. Biol*. 13, 1424-1432.

Payne, R., Murray, D., Harding, S., DB, B., and Soutar, D. (2009) *GenStat for Windows (12th Edition) Introduction*. VSN International, Hemel Hempstead.

Ruello, N. V. (1977) Migration and stock studies on the Australian school prawn *Metapenaeus macleayi*. *Marine Biology* 41, 185-190.

Sahara, K., Marec, F., and Traut, W. (1999) TTAGG Telomeric Repeats in Chromosomes of Some Insects and Other Arthropods. *Chromosome Research* 7, 449-460.

Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sheehy, M. R. J. (2008) Questioning the use of biochemical extraction to measure lipofuscin for age determination of crabs: Comment on Ju *et al.* (1999, 2001). *Marine Ecology-Progress Series* 353, 303-306.

Sheehy, M. R. J., Greenwood, J. G., and Fielder, D. R. (1994) More accurate chronological age determination of crustaceans from field situations using the physiological age marker, lipofuscin. *Marine Biology* 121, 237-245.

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

Uglem, I., Belchier, M., and Svåsand, T. (2005) Age determination of European lobsters (*Homarus gammarus* L.) by histological quantification of lipofuscin. *Journal of Crustacean Biology* 25, 95-99.

Vítková, M., Král, J., Traut, W., Zrzavý, J., and Marec, F. (2005) The evolutionary origin of insect telomeric repeats, (TTAGG)*N*. *Chromosome Research* 13, 145-156.

Vleck, C. M., Haussmann, M. F., and Vleck, D. (2003) The natural history of telomeres: tools for aging animals and exploring the aging process. *Experimental Gerontology* 38, 791-795.

Vogt, G. (2008) How to minimize formation and growth of tumours: Potential benefits of decapod crustaceans for cancer research. *International Journal of Cancer* 123, 2727-2734.

von Zglinicki, T., and Martin-Ruiz, C. M. (2005) Telomeres as Biomarkers for Ageing and Age-Related Diseases. *Current Molecular Medicine* 5, 197-203.

Wahle, R. A., and Fogarty, M. J. (2006) Growth and Development: Understanding and modelling growth variability in lobsters. In: *Lobsters: biology,*

management, aquaculture and fisheries, pp. 1-43. Ed. by B. F. PHILLIPS. Blackwell Publishing, Oxford UK.

Watson, J. (1972) Origin of concatemeric T7 DNA. *Nature New Biology* 239, 197-201.

Zheng, J., Murphy, M. C., and G.H., K. (1995) A length-based population-model and stock-recruitment relationships for red king crab *Paralithodes camtschaticus* in Bristol Bay Alaska. *Canadian Journal of Fisheries and Aquatic Sciences* 52, 1229-1246.

Table 1: Details of crustacean sample groups, number in each group and available morphometric information.

Species	Source	Size class (Number/size class)	Carapace length (mm)	Weight (g)	Sampled Tissues
Metapenaeus macleayi	Lake Wooloweyah near Yamba, New South Wales (29°29'S 153°18'E)	Small (N = 15)	10-14	Unknown	Abdominal tissue minus exoskeleton
		Medium (N = 16)	16-23	Unknown	Abdominal tissue minus exoskeleton
	Yamba vicinity New South Wales offshore (approx 29°26'S 153°22'E).	Large (N = 19)	16-25	Unknown	Abdominal tissue minus exoskeleton
Sagmariasus verreauxi	Waters off Port Hacking, New South Wales (34°17'S 151º18'E) then grown at Cronulla Fisheries, New South Wales	Puerulus (recently settled) (N = 6)	11-15	0.6 -1.4	Abdominal tissue minus exoskeleton
		Small (approx. 3 years old) (N = 5)	65-87	158-364	Muscle and gill
	Coffs Harbour, New South Wales (30°18'S 153°9'E)	Large (N = 5)	103-130	670-1150	Muscle and gill
	Coffs Harbour, New South Wales (30°18'S 153°9'E)	Very large (N = 6)	150-159	>1800	Muscle and gill
Jasus edwardsii	Eastern Tasmanian waters (41°52'S 148°18'E)	Puerulus (recently settled) (N = 7)	unknown	Unknown	Abdominal tissue minus exoskeleton
	Sandstone Bluff area, Tasmania (42°20'S 148°13'E)	Adult (N = 9)	80-120	Unknown	Muscle
Ranina ranina	Caloundra vicinity offshore, Queensland (26°14'S 153°10' E)	Small to large (N = 18)	70-140	Unknown	Muscle and gill

Table 2: Summary of modifications to the *TeloTAGGG* Telomere Length Assay kit for successful TRF assays on crustacean species. Successful TRFs were not achieved for *Ranina ranina* using any of the tested experimental conditions.

Species	Cell Lysis conditions	Restriction Endonucleases	Electrophoresis	Gel
Metapenaeus macleayi	50°C 3 hours	*Hinf*I and *Rsa*I 37°C, 3 hours	Conventional 35V 20 hr 4°C	0.8% agarose in 1 xTAE buffer
Sagmariasus verreauxi	37°C Overnight	*Alu*I and *Hpy*CH4V 37°C, 3 hours	Fluctuating inverted field gel electrophoresis – program optimized for 25-50 kb fragments (Fwd 180 V, Rev 120 V) Switch time 0.4-0.8 linear shape. Recirculating buffer, with run time 20 hr.	1.0 % agarose in 0.5 x TBE buffer
Jasus edwardsii	50°C 3 hours	*Alu*I and *Hpy*CH4V 37°C, 5 hours	Conventional 35V 20 hr 4°C	0.8% agarose in 1 xTAE buffer

Table 3: Mean and standard errors (SE) for telomere length estimates from different tissues and size classes of *Sagmariasus verreauxi*.

Tissue	Telomere length (bp) Mean ± SE
Puerulus – tail (Abdomen – exoskeleton)	17908 ± 366
Adult gill	17603 ± 565
Adult muscle	16318 ± 486
Adult gill muscle combined	16961 ± 1131
Size class	
Small (3 years old)	16720 ± 618
Large	18190 ± 785
Very Large	15961 ± 630

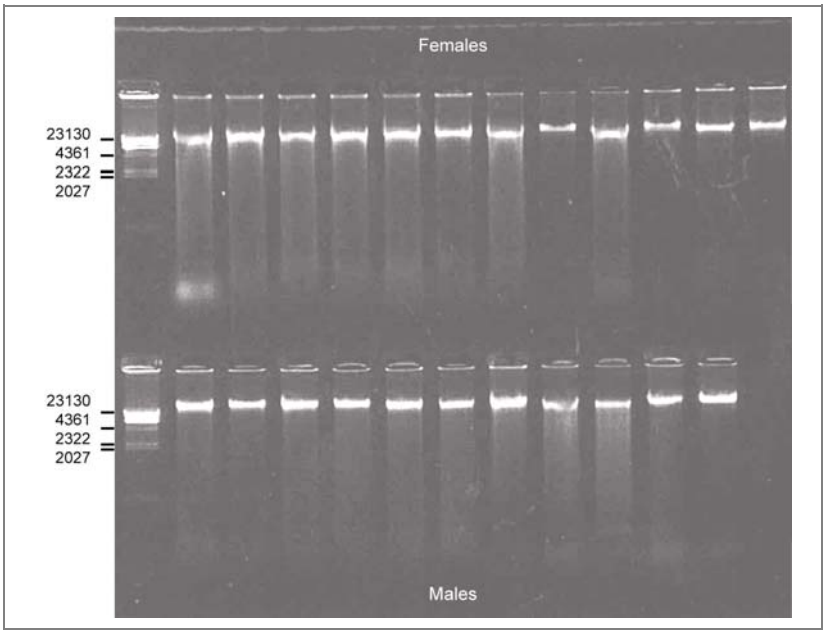

Figure 1: High quality uncut genomic DNA from 12 female and 11 male medium sized *Metapenaeus macleayi* which were subsequently used for telomere length assays. Molecular weight markers (bp) are shown on the left hand side.

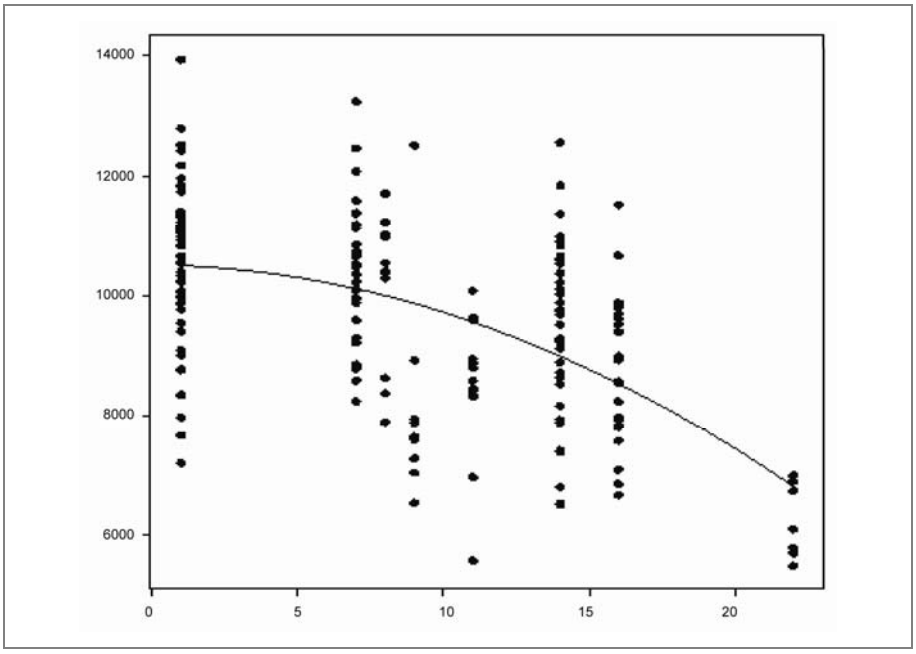

Figure 2: The effect of DNA storage time (between extraction and TRF analysis: in days), on telomere length values (in bp) of *Metapenaeus macleayi*.

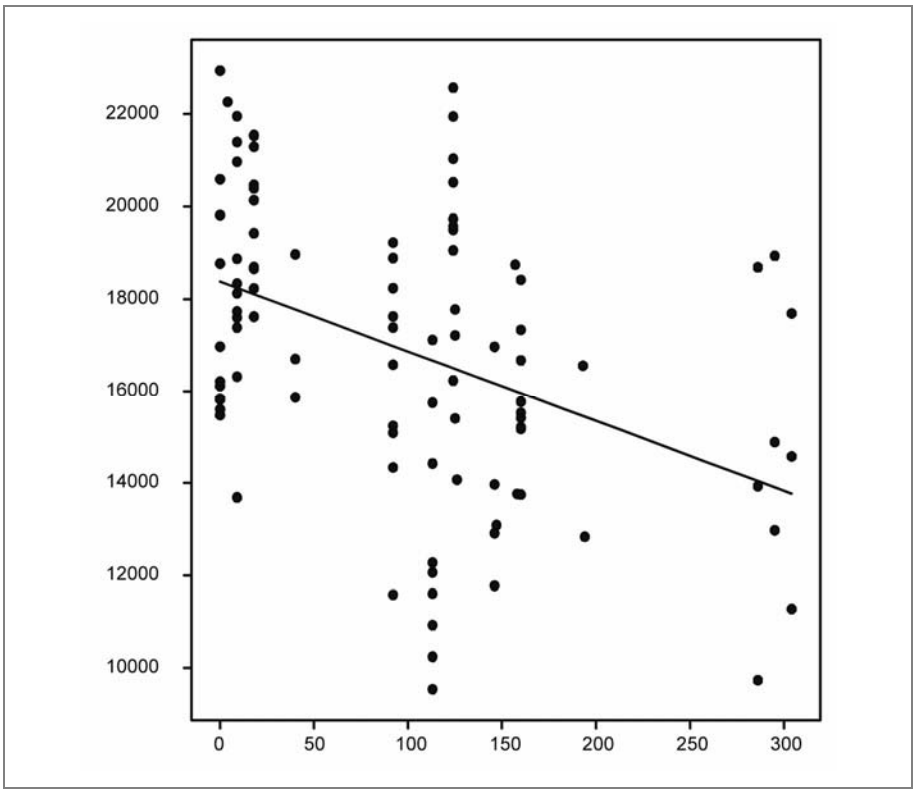

Figure 3: The effect of DNA storage time (between extraction and TRF analysis: in days), on telomere length values (in bp) of *Sagmariasus verreauxi.*

Appendix 5.4 – Submitted Manuscript

Telomere biology in a freshwater fish, *Bidyanus bidyanus* (silver perch)

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Abstract

The age of individuals in wild populations helps scientists understand the biology and life-history of different species. Unlike the situation in humans, obtaining accurate age data for wild animals is often problematic. The traditional way of gathering age data for bony fishes is to determine the number of growth rings from sectioned otoliths (also known as 'ear stones'). This technique is destructive and therefore is not appropriate for use to age rare or vulnerable species such as *Bidyanus bidyanus* (silver perch). Telomeres are nucleoprotein structures at the ends of linear eukaryotic chromosomes, which prevent aberrant recombination and degradation of the chromosomal ends. In humans and mice telomere length typically shortens after each mitotic cell division over the life of an animal. It has been suggested that this shortening may provide a molecular biomarker for estimating age in other species. In this study, the relationship between telomere length and chronological age was tested in *B. bidyanus*, and the variability of telomere length with regard to different tissue types was examined. There was a significant shortening in telomere length between two and three-year-old fish, which was consistent in all three tissues (heart, skeletal muscle and liver). Telomere length was significantly different among the three tissues tested (heart 3.5 kb, liver 3.7 kb and skeletal muscle 4.2 kb). These findings are consistent with most previous studies in human and mouse. Larger sample sizes with more age groups and different tissues, and longitudinal studies on individuals are required to further validate this experimental approach as a tool for age-estimation in the study of *B. bidyanus*.

Keywords

DNA, TRF, Age estimation, Australia

Introduction

Telomeres are the DNA sequences and proteins located at the ends of eukaryotic chromosomes (von Zglinicki, 2002). The telomeric DNA of most vertebrates is composed of repetitive DNA sequences of TTAGGG (Allshire *et al.* 1989). This sequence has been conserved throughout evolution reflecting its important role in preventing chromosome degradation and maintaining genome integrity (Meyne *et al.* 1989).

Telomeres provide the cell with a mechanism to distinguish between real chromosome ends and those arising from chromosome breaks that need to be repaired. Due to the end replication problem, telomere repeat units are lost at each cell division (Watson, 1972). The replication process can cause a loss of nucleotide sequences between 3 and 150 base pairs for each cell division depending on the species (Chan and Blackburn, 2003). As telomeres gradually shorten throughout the aging process, telomere length has been developed as one of the genetic tools for age determination (Hall *et al.* 2004; Haussmann *et al.* 2003).

Telomeres are thought to shorten in length at a constant rate, and studies in several organisms, including birds, dogs, rodents and humans, have shown there is some correlation between age and

average telomere length in at least some tissues (Haussman *et al*. 2002; Hiyama *et al.* 1996; Takubo *et al.* 2002). However, the rate at which telomere repeats are lost can vary between tissues as cell turnover rates are not necessarily the same (Forsyth *et al.* 2002).

Bidyanus bidyanus, Mitchell 1938 (Terapontidae), is endemic to the Murray-Darling river system. The fish has been recognised for its potential for aquaculture, and hatchery techniques are well established (Rowland, 2009). Studies of aging and growth of wild *B. bidyanus* involves sectioning and reading otoliths. These studies have confirmed that the incremental annuli observed on the otolith sections are formed annually up to ten years of age (Mallen-Cooper and Stuart, 2003). However, this destructive method, which involves killing fish to remove otoliths, is undesirable (Beamish and Neville, 1992). The possible application of telomere length analysis in relation to *B. bidyanus* aging may overcome this problem by providing an efficient and non-destructive tool of age determination.

This project examined the possibility of using telomere length as a biomarker for studying aging in *B. bidyanus*. The objectives were to determine telomere length across age groups and whether or not telomere length varies in different tissues of this species. *Bidyanus bidyanus* were sourced from the New South Wales Department of Primary Industries' Grafton Aquaculture Centre and encompassed four age classes ranging from three months to three years.

Materials and Methods

Samples and telomere length estimation

Fish were euthanased using the anaesthetic Benzocaine following procedures outlined by Barker and others (2009). Entire heart, liver and a portion of the left abdominal wall muscle tissue (without skin) were dissected from one, two and three-year-old fish. The entire body was sampled from three-month-old juvenile fish, excluding the head. Genomic DNA was extracted from frozen tissue using standard methods (Sambrook and Russell, 2001). Telomere length was measured using the *T*elo*TAGGG* Telomere Length Assay kit (Roche Diagnostics Australia Pty. Ltd., Castle Hill NSW, Australia) with minor modifications as follows; genomic DNA (2 µg) was digested with restriction endonucleases *RsaI* and *HinfI* for three hours at 37°C and the resulting DNA fragments were separated by gel electrophoresis on a 1% non-denaturing agarose gel at 35 V for 20 hours at 4°C. The DNA was then nicked in-gel at 60 mJ UV before denaturation and neutralisation. Subsequent transfer of the DNA to nylon membrane, hybridisation to the telomere-specific probe, chemiluminescence and detection were according to the Roche *T*elo*TAGGG* kit instructions. Chemiluminescent signals were captured on x-ray film using multiple exposures as appropriate.

Data analysis

The x-ray film was scanned and the average TRF length of each sample was calculated by densitometric analysis of x-ray film using Quantity One software (Biorad Australia, Figure 1). Mean TRF was calculated using the formula stated by the *T*elo*TAGGG* telomere length assay (Roche Diagnostics Australia),

$$\text{Mean TRF} = \sum (OD_i) / \sum (OD_i / L_i)$$

where OD_i is the total chemiluminescence signal and L_i is the length of the TRF fragment at position 'i'. The telomere length assay was repeated three times for each tissue sample and samples were loaded in random order on every gel.

Statistical analyses were conducted using GenStat (Payne *et al.* 2009) using one-way ANOVA. The dependent variable in this experiment was telomere length, and independent factors were the age groups and tissue types. A split-plot design was used for different tissues and replicate measurements of the same individuals. If the ANOVA indicated a significant ($P<0.05$) difference, Fisher's Least-significance difference (LSD) test was used to compare means.

Results

Telomere length variation between tissue types

Telomere length variability between tissue types was examined using the three-year-old *B. bidyanus*. The mean telomere lengths of the three tissues (heart, liver and muscle) were found to be different within the age group ($P=0.03$). The shortest telomeres were in the heart samples with a length of 3,531 bp, followed by the liver with a mean length of 3,769 bp. The longest mean telomere length was observed from the sample of the skeletal muscle, which had 4,249 bp (Table 1). The telomere length of heart and muscle were significantly different from one another (Fisher's LSD = 517, $P=0.05$) whereas the liver tissue was intermediate and did differ significantly from the other two tissue types (Table 1).

Telomere length variation across ages for the same tissue types

The change in telomere length with age from one to three years was studied for each tissue separately. There was a significant effect of age on

telomere length for all three tissues (Table 2). For all three tissue types, a similar trend was observed whereby the telomere length of three-year-old fish was significantly shorter than that of two-year-old fish but not one-year-old fish (Table 2). In other words, two-year-old telomere length (TL) was slightly higher than one or three-year-old fish. This trend was most pronounced in liver tissue and least pronounced in skeletal muscle.

The telomere length of three-month-old fish was also measured; however, this measurement was based on a mixture of tissues from the whole body and was therefore excluded from the tissue-by-tissue analysis of the other age groups. A mean telomere length of 3,590 bp with standard deviation of 407 bp and coefficient of variation of 11% was recorded for three-month-old fish. This average telomere length was similar to the other age classes measured. It was significantly shorter than the telomere lengths of all three tissues of the two-year-old but not the one or three-year-old fish.

Discussion

Measuring TL could potentially be a quicker, relatively non-invasive method to estimate the age of individuals within a population once a relationship has been determined for that specific population (Haussmann and Vleck, 2002). Telomere shortening used to be thought to occur at a constant rate as several studies have found some correlation between telomere length and age (Allsopp *et al.* 1992; Hastie *et al.* 1990; Slagboom *et al.* 1994). However, results with *B. bidyanus* agree with other studies that indicate that telomere shortening is not just caused by simple cell division and there is often substantial unexplained variation in the relationship between telomere length and age (Nakagawa *et al.* 2004).

The TRF method used in this project was successful in estimating mean telomere length in *B. bidyanus* with an acceptable degree of precision. Unlike some other methods, such as fluorescence in situ hybridisation (FISH), which only focuses on measuring telomere length on specific chromosomes, TRF averages the length across all chromosomes and cells in a particular tissue type. Recent evidence suggests that telomere lengths on groups of chromosomes, rather than on single chromosomes, are important in cell death (Hemann *et al.* 2001). Hence, TRF method is suitable as it is not limited to only showing the rate of loss in a single cell and also it is not subject to inter-chromosomal variability in telomere length (Monaghan and Haussmann, 2006).

In this study TL was found to vary significantly between tissue types (Table 1). Tissues can differ in their telomere length because of the different cell turnover rates in each tissue. Telomere lengths and their rate of change vary between chromosomes, cells, tissues and individuals within the same age (Monaghan and Haussmann, 2006). One explanation is that the rate of cell divisions varies between tissues. Generally, tissues can be categorised into groups that are, (1) post-mitotic after reaching mature stage, or (2) continually requiring high levels of cell proliferation. With tissues that are predominantly mitotically inactive, such as skeletal muscle and brain, the rate of change of telomere length will be fairly stable with limited cell division (Takubo *et al.* 2002). Conversely, other tissue types such as intestinal mucosa and bone marrow, containing a high proportion of stem cells, have rapid turnovers and proliferate through life (Haussmann *et al.* 2002; Hiyama *et al.* 1996). Other tissues, such as the liver and renal cortex, show telomere shortening with age, despite little mitotic activity (Djojosubroto *et al.* 2003).

When the telomere length was compared in three tissues within one age group of *B. bidyanus*, the largest difference in TL between tissues was found between heart and skeletal muscle. The variation in TL between tissues types has also been observed in other studies. Longer telomere lengths were observed in muscle tissue than in skin tissue of the short lived fish species *Nothobranchius furzeri* (Hartmann *et al.* 2009). In a study of mangrove snapper (*Lutjanus argentimaculatus*), Ying (2005) showed that mean TL varied between brain, muscle and blood tissues. Telomere length decreased with age for blood and muscle tissue but the rate of telomere shortening was fastest for blood and slowest for muscle. Unlike mammals, many fish, including *B. bidyanus* are observed to have white muscle tissue with very low levels of perfusion and red cardiac muscles which contains a relatively high proportion of blood. The physiology of fish may therefore explain the observation of two muscle types (heart and body muscle) in *B. bidyanus* having a significant difference of TL.

Another study on telomere attrition with ageing in Wistar rats (*Rattus norvegicus*) also found differences between five tissues they sampled (Hastings *et al.* 2004). However, this difference in TL between tissue types was not observed in neonates. Therefore it can be expected that TL in three tissues of *B. bidyanus* may show a greater differences once the fish matures (Mallen-Cooper and Stuart, 2003).

In this study the change in telomere length with age was examined in three tissues and significant differences between three age groups were

observed (Table 2). Telomere length decreased between two and three years of age and the trend was most pronounced in liver samples. The results of three-month-old *B. bidyanus* was excluded in this tissue-by-tissue analysis for age classes as the analyses were performed on a mixture of tissues. The analysis also showed there was a slight increase in the TL between one and two-year-old fish. This may be related to elevated levels of telomerase activity of the cell. Telomerase has an ability to counteract the shortening of telomeres by generating additional nucleotides and attaching them to the end of telomere (Greider and Blackburn, 1985; 1987). Telomerase appears to be most active in younger ages; hence, this may explain why telomere lengths were at least maintained between one and two years of age (Bodnar *et al.* 1998; Counter *et al.* 1998; Taylor and Delany, 2000).

Empirical results from a study on mangrove snapper showed a negative relationship between telomere length and age of the fish across three tissues types (Ying, 2005). Other vertebrate studies, including those on human and birds have also reported similar patterns (Haussmann *et al*. 2003). Despite many studies that have found telomere length shortens with increasing age, some studies with Leach's storm-petrels (*Oceanodroma leucorhoa*) and captive black sea bream (*Acanthopagrus schlegeli*) have shown positive relationships between telomere length and age (Vleck *et al.* 2003; Ying, 2005) and in *Danio rerio* no appreciable shortening of telomeres was observed with age in any tissue (Lund *et al.* 2009). Interestingly, unlike the results found on a captive black sea bream, studies of wild caught black sea bream and European sea bass found no correlation between blood telomere length and age (Horn *et al.* 2008; Ying, 2005). In order to make a valid comparison of the relationship between TL and age of *B. bidyanus* with other species, further research with wider age ranges and growth conditions will be necessary as the fish samples in the current study only represented third of the life span of the species and were drawn from captive-bred populations.

Several studies have found that telomeres are sensitive to reactive oxygen species damage and the rate of telomere loss is dependent on providing telomere protection between oxidative stress and antioxidant defence (Jennings *et al*. 1999; 2000; Metcalfe and Monaghan, 2001; 2003; von Zglinicki, 2002). The breeding regime of the aquaculture population of *B. bidyanus* make it likely that each age group was kept separately with similar rearing conditions, such as temperature, feeding regime or stock density (Rowland, 2009). However, stress response can not be excluded as the environment was not completely alike. Since the three-year-old *B. bidyanus* were collected one year later than all other sample groups, there is a possibility that the different cohorts could have been exposed to different levels of oxidative stress.

Apart from the potential stress response, the influence of genetic factors on telomere length of *B. bidyanus* could not be excluded. It was not known if the fish samples in this study were representative of the genetic diversity of wild *B. bidyanus*, and the potential exists that the test specimens may have bred from a small broodstock. If so, they may be expected to show a smaller genetic variance and therefore have similar telomere length at birth than fish in a natural population.

This study demonstrated the telomere lengths of *B. bidyanus* shortened across ages, and telomere length differed between all three tissues tested. It also found that the relationship of telomere length of change with age was consistent between tissue types. Further improvement could be made to results by employing a larger number of samples with a wider age distribution. It would be interesting to examine a greater variety of tissues that are known to have different cell turnover rates. Q-PCR analysis (Nakagawa *et al*. 2004) for studying telomere length is also worthwhile investigating as the method requires smaller quantities of DNA, and samples can be obtained from blood or fin-clips. Longitudinal studies on fish samples are recommended to directly test whether telomere lengths changes over the lifetime of an individual. This would allow the impact of stress levels on the length of telomeres to be measured. Finally, before implementing telomere length to estimate age of *B. bidyanus*, the rate of change of telomere length for the species needs to be identified. Should telomere length be adopted as an age biomarker for *B. bidyanus*, it will be essential to validate the method against the existing otolith aging technique.

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References

Allshire, R. C., Dempster, M. and Hastie, N. D. (1989) Human telomeres contain at least three types of G-rich repeat distributed non-randomly. *Nucleic Acids Research* 17, 4611-4627.

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

Barker, D., Allan, G. L., Rowland, S. J., Kennedy, J. D. and Pickles, J. M. (ed.) (2009) *A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research*: New South Wales Department of Primary Industries and Fisheries.

Beamish, R. J. and Neville, C. E. M. (1992) The importance of size as an isolating mechanism in lampreys. *Copeia*, 191-196.

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

Chan, S. W. L. and Blackburn, E. H. (2003) Telomerase and ATM/Tel1p protect telomeres from nonhomologous end joining. *Molecular Cell* 11, 1379-1387.

Counter, C. M., Hahn, W. C., Wei, W. Y., Caddle, S. D., Beijersbergen, R. L., Lansdorp, P. M., Sedivy, J. M. and Weinberg, R. A. (1998) Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. *Proceedings of the National Academy of Sciences of the United States of America* 95, 14723-14728.

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

Forsyth, N. R., Wright, W. E. and Shay, J. W. (2002) Telomerase and differentiation in multicellular organisms: Turn it off, turn it on, and turn it off again. *Differentiation* 69, 188-197.

Greider, C. and Blackburn, E. (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell* 43, 405-413.

Greider, C. W. and Blackburn, E. H. (1987) The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* 51, 887-898.

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

Hartmann, N., Reichwald, K., Lechel, A., Graf, M., Kirschner, J., Dorn, A., Terzibasi, E., Wellner, J., Platzer, M., Rudolph, K. L., Cellerino, A. and 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. and Allshire, R. C. (1990) Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 346, 866-868.

Hastings, R., Li, N. C., Lacy, P. S., Patel, H., Herbert, K. E., Stanley, A. G. and Williams, B. (2004) Rapid telomere attrition in cardiac tissue of the ageing Wistar rat. *Experimental Gerontology* 39, 855-857.

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

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

Haussmann, M. F., Winkler, D. W., O'Reilly, K. M., Huntington, C. E. and Vleck, C. M. (2002) Telomere shortening in birds varies with life span, pp. 1241-1242.

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

Hiyama, E., Tatsumoto, N., Kodama, T., Hiyama, K., Shay, J. W. and Yokoyama, T. (1996) Telomerase activity in human intestine. *International Journal of Oncology* 9, 453-458.

Horn, T., Gemmell, N. J., Robertson, B. C. and Bridges, C. R. (2008) Telomere length change in

European sea bass (Dicentrarchus labrax). *Australian Journal of Zoology* 56, 207-210.

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

Jennings, B. J., Ozanne, S. E. and 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.

Lund, T. C., Glass, T. J., Tolar, J. and 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.

Mallen-Cooper, M. and Stuart, I. G. (2003) Age, growth and non-flood recruitment of two potamodromous fishes in a large semi-arid/temperate river system. *River Research and Applications* 19, 697-719.

Metcalfe, N. B. and Monaghan, P. (2001) Compensation for a bad start: grow now, pay later? *Trends in Ecology* and *Evolution* 16, 254-260.

Metcalfe, N. B. and Monaghan, P. (2003) Growth versus lifespan: perspectives from evolutionary ecology. *Experimental Gerontology* 38, 935-940.

Meyne, J., Ratliff, R. L. and Moyzis, R. K. (1989) Conservation of the Human Telomere Sequence (TTAGGG)n among Vertebrates. *Proceedings of the National Academy of Sciences of the United States of America* 86, 7049-7053.

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

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

Payne, R., Murray, D., Harding, S., DB, B. and Soutar, D. (2009) *GenStat for Windows (12th Edition) Introduction*. Hemel Hempstead: VSN International.

Rowland, S. J. (2009) Review of Aquaculture Research and Development of the Australian Freshwater Fish Silver Perch, Bidyanus bidyanus. *Journal of the World Aquaculture Society* 40, 291-324.

Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Slagboom, P. E., Droog, S. and Boomsma, D. I. (1994) Genetic determination of telomere size in humans - A twin study of 3 age groups. *American Journal of Human Genetics* 55, 876-882.

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

Taylor, H. A. and Delany, M. E. (2000) Ontogeny of telomerase in chicken: Impact of downregulation on pre- and postnatal telomere length in vivo. *Development Growth* and *Differentiation* 42, 613-621.

Vleck, C. M., Haussmann, M. F. and Vleck, D. (2003) The natural history of telomeres: tools for aging animals and exploring the aging process. *Experimental Gerontology* 38, 791-795.

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

Watson, J. (1972) Origin of concatemeric T7 DNA. *Nature New Biology* 239, 197-201.

Ying, T. C. (2005) Evaluation of telomere length as an age-marker in marine species, vol. Master of Philosophy, pp. 185. Hong Kong: University of Hong Kong.

Table 1: Statistical results generated by the one-way ANOVA for the comparisons between tissue types of the three-year-old *Bidyanus bidyanus*. The bottom half of the table presents telomere length means (bp) and standard deviations (SD) of three-year-old *B. bidyanus* for three different tissues (n = 7). Different letters in parenthesis (a, b) indicate significant differences between tissue means (P<0.05).

	Statistic	
One–way ANOVA	$F_{2,23} = 4.29$; P = 0.03	
Standard errors of means	177	
Fisher's LSD P = 0.05	517	
CV between individual fish	17%	
CV between replicates	26%	
	Mean ± S.D.	Coefficient of variation
Heart	3531 ± 228[a]	6%
Liver	3769 ± 698[ab]	15%
Muscle	4249 ± 1132[b]	27%

Table 2: Statistical results generated by the one-way ANOVA for the effect of age on telomere length within tissue for *Bidyanus bidyanus*. The bottom half of the table presents telomere length means (bp) and standard deviations (SD) of three age groups of *B. bidyanus* samples for three different tissues (n = 7). Different letters in parenthesis (a, b) indicate significant differences between age means (P<0.05).

	Statistic		
One-way ANOVA	$F_{2,15} = 4.44$; P = 0.03	$F_{2,14} = 6.08$; P = 0.01	$F_{2,15} = 5.53$; P = 0.02
Standard errors of means	130	140	89
Fisher's LSD P = 0.05	393	424	268
CV% between replicates	6%	11%	9%
CV% between animals	15%	16%	10%
Age	Heart	Liver	Muscle
1	3878 ± 340[ab]	3696 ± 447[ab]	3680 ± 314[ab]
2	4022 ± 487[a]	4116 ± 554[a]	3940 ± 377[a]
3	3491 ± 237[b]	3432 ± 379[b]	3525 ± 243[b]

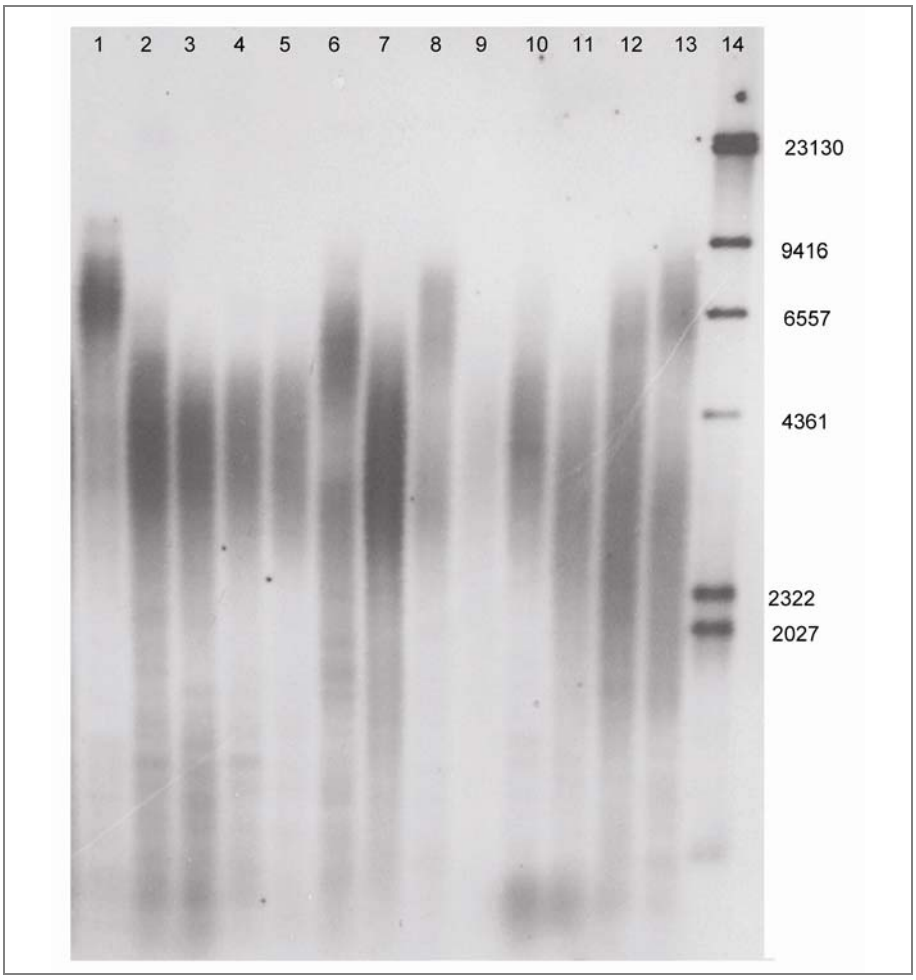

Figure 1: A representative autoradiograph for telomere length analysis of silver perch. 1, 2, 6, 8, 12 and 13 are two-year-old heart samples; 10 is one-year-old heart sample; 3, 4, 5, 7, 9 and 11 are three-month-old body samples; 14 is the λ HindIII molecular weight marker where sizes are shown in base pairs (bp).