

Investigation of the molecular
ageing process of the long-lived
bivalve *Arctica islandica*

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Summary

The question on why we age and how ageing proceeds has occupied researchers' minds for a long time. Demands on research of healthy ageing and geriatric diseases rise with an older growing human population. Thus, studying the mechanisms of ageing in animals with extraordinarily long lifespans could possibly reveal secrets to longevity and healthy ageing.

In this study, a short-lived population of the bivalve mollusk *Arctica islandica* from the Baltic Sea (with a maximum lifespan – MLSP – of 40 years) was compared to a long-lived population from Iceland (MLSP >400 years) with respect to physiological and molecular damage parameters and gene expression. Both populations originate from two very distinct environments that may influence the ageing process of the animals. Cohorts of both populations were sampled in 2010 and sexes and ages of all animals were determined. Based on this data, ageing markers (oxidation of proteins, lipids, and nucleotides, protein stability, telomere length, telomerase activity) from selected individuals along sampled age ranges (10-36 years in the Baltic Sea and 6-226 years in Iceland) of the two populations were investigated. Oxidation to nucleotides significantly accumulated over age in both populations and did so significantly faster in the shorter-lived Baltic Sea compared to the longer-lived Iceland population regarding their absolute chronological age. Remarkably, when looking at the results in respect to the relative lifespan of each population, nucleotide damage accumulated at the same pace in both populations. Additionally, a significantly higher mean level of DNA damage was detected in the shorter-lived Baltic Sea population where frequent hypoxic and anoxic events occur and the animals are living in a warmer environment. Interestingly, significantly higher mean levels of lipid peroxidation were observed in the long-lived cold-adapted Iceland population that might result from a different lipid composition at colder temperatures. Lipid peroxidation over investigated ages, however, stayed stable in both populations. All other investigated parameters also stayed stable over age in the two populations, which points towards a remarkable molecular stability, exceptional repair or regeneration capacity of *A. islandica*.

Parallel to the ageing-marker investigations, mRNA of selected, different old animals was sequenced using 454 pyrosequencing to represent combined transcriptomes

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from post-mitotic heart and highly proliferating gill tissue of different age groups of the two populations. This transcriptome database was used to analyze global gene expression profiles over age of the two populations but also to identify genes of interest for further detailed gene expression analysis via qRT-PCR. The transcriptome analyses revealed a distinct gene expression behavior in young Iceland individuals compared to older age groups and hence, a specific transcriptional pattern for older age classes could not be defined. Together with findings from other *A. islandica* studies and considering the shape of the observed growth curve, a different physiology in young compared to middle and old aged animals can be revealed up to approx. 40 years of age. Since this is the lifespan of the Baltic Sea *A. islandica* animals, two different life strategy of both populations could have developed that remain unclear, but factors beneficial for a long life may only be selected for in the longer-lived Iceland population since reproductive output in older/larger animals is much higher than in younger ones.

Due to the accumulation with age and immense detected differences in oxidative damage to nucleotides between the two populations, gene expression over age of identified DNA repair genes from the RNAseq database were analyzed with qRT-PCR. Further, the expression of antioxidants playing a role in the oxidative stress response, that have previously been observed to be differentially expressed between *A. islandica* populations under stressful conditions, and that are proposed to be relevant in ageing according to the free radical theory of ageing (Harmann, 1956), was investigated in qRT-PCR. At last, gene expression over age in the two populations with distinct MLSPs was also investigated in known ageing-associated genes. For all analyzed genes a stable expression over complete sampled lifespan (10-36 years for Baltic Sea and 6-226 years for Iceland population) was detected in both populations. Significant differences in mean gene expression levels between the short- and long-lived population, however, could be observed for several DNA repair, ageing-associated, and antioxidant coding genes. Neither a better DNA repair nor oxidative stress capacity, however, in neither of the two populations could be revealed but hints towards a differentially evolved gene expression behavior in investigated populations. Extreme different environmental factors may evoke epigenetic changes in the populations' genomes that lead to differential gene expression that might further be reflected in the populations' specific MLSPs. Further investigation on environmentally induced changes on the epigenome and dependent gene expression of differentially expressed genes of interest identified in this study and further investigations on the protein levels in these two populations may be the next step leading to identify ways for healthy ageing.

Zusammenfassung

Die Frage warum und wie wir altern beschäftigt Forscher schon seit langer Zeit. Mit einer stetig älter werdenden Bevölkerung steigt der Bedarf an Forschung auf den Gebieten „Gesundes Altern“ sowie Alterskrankheiten. Die Untersuchung von Alterungsmechanismen in Tieren mit außergewöhnlich langer Lebensspanne könnte daher Geheimnisse zur Langlebigkeit oder dem gesunden Altern aufdecken.

In dieser Studie wurden daher zelluläre physiologische und molekulare Schäden sowie die Genexpression einer kurzlebigen Population der Muschel *Arctica islandica* aus der Ostsee (maximale Lebensspanne – MLSP – 40 Jahre) mit einer langlebigen Population von Island (MLSP >400 Jahre) verglichen. Beide Populationen stammen aus zwei Habitaten mit sehr unterschiedlichen Umweltbedingungen, die den Alterungsprozess der Tiere beeinflussen könnten. In 2010 wurden Kohorten der beiden Populationen gesammelt und das Alter sowie das Geschlecht aller Tiere bestimmt. Basierend auf diesen Daten wurden Individuen beider Populationen entlang der gesammelten Altersspanne (10-36 Jahre in der Ostseepopulation und 6-226 Jahre in der Islandpopulation) selektiert und deren Altersmarker (Oxidation von Proteinen, Lipiden und Nukleotiden, Proteinstabilität, Telomerlängen, Telomeraseaktivität) untersucht. Die Oxidation von Nukleotidsäuren stieg in beiden Populationen über das absolute chronologische Alter signifikant an, was zusätzlich in der kurzlebigen Ostseepopulation signifikant schneller erfolgte als in der langlebigen Islandpopulation. Betrachtet man die Anreicherung der DNA und RNA Schäden hinsichtlich der relativen Lebensspanne der beiden Populationen so stieg dieser Parameter bemerkenswerterweise mit der gleichen Geschwindigkeit an. Zusätzlich wurde ein signifikant höheres Niveau an DNA Schäden in der kurzlebigen Ostseepopulation festgestellt, in deren Habitat häufig hypoxische und anoxische Umweltbedingungen und höhere Temperaturen vorherrschen. Interessanterweise wurden in der langlebigen, kalt-adaptierten Islandpopulation signifikant höhere Durchschnittswerte für die Lipidperoxidation ermittelt, was von unterschiedlichen Lipidzusammensetzungen bei kälteren Temperaturen herrühren kann, das Niveau oxidiertes Lipide blieb jedoch über die gesamte Lebensspanne stabil. Alle anderen untersuchten Altersmarker verhielten sich in beiden Populationen über das Alter ebenfalls stabil, was

auf eine bemerkenswerte molekulare Stabilität, außergewöhnliche Reparatur- oder Regenerationskapazität in *A. islandica* hinweist.

Zusätzlich zu oben genannten Untersuchungen wurden kombinierte Transkriptome aus post-mitotischem Herzgewebe und stark proliferierender Kieme selektierter Tiere unterschiedlichen Alters aus beiden Populationen mittels 454 Pyrosequenzierung generiert. Diese Transkriptomdatenbank wurde für globale Genexpressionsanalysen über das Alter beider Populationen und zur Identifikation geeigneter Kandidatengene, für weitere detaillierte Genexpressionsanalyse mittels qRT-PCR, genutzt. In der Transkriptomanalyse fiel auf, dass sich das Genexpressionsverhalten der jungen Isländischen Individuen von den älteren Altersgruppen unterschied und somit kein spezifisches transkriptionelles Muster älterer Tiere definieren ließ. Unter Berücksichtigung anderer *A. islandica* Studien und der Form der erstellten Wachstumskurve, scheinen junge Tiere bis zu einem Alter von ca. 40 Jahren verglichen mit mittelalten und alten Tieren eine unterschiedliche Physiologie aufzuweisen. Da dies der Lebensspanne von *A. islandica* Tieren aus der Ostsee entspricht, könnten sich zwei bisher unergründete unterschiedliche Lebensstrategien in beiden Populationen entwickelt haben und Faktoren, die ein langes Leben begünstigen, könnten nur in der länger-lebigen Islandpopulation selektiert worden sein, da der Reproduktionsertrag in älteren/größeren Tieren wesentlich höher ist als in jüngeren.

Aufgrund der sich anreichernden Nukleotidoxidation mit zunehmendem Alter und der enormen Unterschiede dieses Faktors zwischen den zwei Populationen, wurde die Genexpression von DNA Reparaturgenen, die in der RNAseq Datenbank identifiziert wurden, mittels qRT-PCR entlang des Alters analysiert. Weiterhin wurde die Expression von Antioxidantien mittels qRT-PCR untersucht, die eine Rolle in der Oxidativen Stress Antwort spielen, und deren unterschiedliche Expression zwischen *A. islandica* Populationen nach Stressexposition bereits festgestellt worden ist, sowie nach der „free radical theory of ageing“ (Harmann, 1956) eine altersrelevante Rolle zugesprochen wurde. Zusätzlich wurde die Genexpression bekannter Alterungsassoziierter Gene in den zwei Populationen mit den verschiedenen MLSPs untersucht. In beiden Populationen wurde eine stabile Expression aller analysierten Gene über die gesamte beprobte Lebensspanne (10-36 Jahre in der Ostsee- und 6-226 Jahre in der Islandpopulation) festgestellt. Signifikante Unterschiede des durchschnittlichen Genexpressionsniveaus zwischen der kurz- und der langlebigen Population konnten für einige DNA Reparaturgene, Alterungs-assozierte und Antioxidantien-kodierenden Gene festgestellt werden. Die Ergebnisse sprechen jedoch weder für eine bessere DNA Reparaturkapazität noch für eine bessere oxidative Stressbewältigung in einer der beiden Populationen. Jedoch könnte dies auf ein unterschiedlich entwickeltes Genexpressionsverhalten der untersuchten Populationen hinweisen. Extrem unter-

Zusammenfassung

schiedliche Umweltfaktoren könnten epigenetische Veränderungen in den Genomen der Populationen hervorrufen, die zu unterschiedlicher Genexpression führen können und sich weitergehend in den populationsspezifischen MLSPs widerspiegeln. Weitere Untersuchungen der umweltbedingt induzierten Veränderungen des Epigenoms und die davon abhängige Genexpression der hier identifizierten unterschiedlich exprimierten Kandidatengene, sowie die Untersuchung beider Populationen auf Proteinebene könnten den nächsten Schritt zu gesunderem Altern darstellen.

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Frequently used Abbreviations

8-oxoGuo	8-oxo-7,8-dihydroguanosine
8-oxodGuo	8-oxo-7,8-dihydro-2'-deoxyguanosine
a.k.a.	also known as
AOX	alternative oxidase
APE1	Apurinic/apyrimidinic (AP) endonuclease 1
Appendix e	electronic appendix
Appendix p	paper appendix
APOE	apolipoprotein E
approx.	approximately
ATP	adenosine triphosphate
BER	base excision repair
BisANS	4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid
bp	base pairs
BS	Baltic Sea
CAT	catalase
cDNA	copy DNA
CT	cycle threshold
Cu/ZnSOD	copper/zinc superoxide dismutase
DBS	double-strand breaks
ddNTP	dideoxynucleotide triphosphate
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase
dNTP	deoxynucleotide triphosphate
ds	double-stranded
EDTA	Ethylenediaminetetraacetic acid
EtOH	ethanol
EXO1	exonuclease 1
F ₂ -IsoPs	F ₂ -isoprostanes
FEN1	flap endonuclease 1
FTC	fluorescein-5-thiosemicarbazide

Frequently used Abbreviations

FOXO3A	transcription factor: forkhead box O3A
<i>g</i>	gravitational force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene Ontology
GPx	glutathione peroxidase
H ₂ O	tap water
HCl	hydrochloric acid
HPLC-ECD	high-performance liquid chromatography-electro-chemical detection
HR	homologous recombination
IC	Iceland
ID	identifier
kb	kilo base-pairs
KEGG	Kyoto Encyclopedia of Genes and Genomes
KPO ₄	potassium phosphate
LIG	ligase
LSG	line of strongest growth
M	molar
Mb	mega base-pairs
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulfate
MLSP	maximum lifespan
MMR	mismatch repair
MnSOD	manganese superoxide dismutase
MRD	metabolic rate depression
mRNA	messenger RNA
MSH6	mutS homologue 6
NaCl	sodium chloride
NaOCl	sodium hypochlorite
NBS1	Nijmegen breakage syndrome 1
NHEJ	non-homologous end-joining
NER	nucleotide excision repair
NGS	next-generation sequencing
PCR	polymerase chain reaction
POL β	DNA polymerase β
PP _i	pyrophosphate
ppt	parts-per-trillion, 10 ⁻¹²
PTP	picotiter plate
qRT-PCR	quantitative realtime PCR

Frequently used Abbreviations

RNA	ribonucleic acid
RNAseq	RNA sequencing
ROS	reactive oxygen species
RPKM	reads per kilobase per million mapped reads
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulphate
SIRT1 / 2 / 6	sirtuin 1 / 2 / 6
SOD	superoxide dismutase
ss	single-stranded
SSB	single-strand breaks
T-ACE	transcriptome analysis and comparison explorer
TAE	Tris-acetate-EDTA buffer
UV	ultra violet
w/v	weight per volume
WRN	Werner syndrome, ATP-dependent helicase
XRCC1 / 4	X-ray repair cross-complementing protein 1 / 4

1 Introduction

1.1 What is ageing?

Many attempts have been made to define ageing. Ageing as “any change in an organism over time” has been described by Bowen *et al.* [27] who besides considering functional decline over age *i.e.* senescence, also incorporate positive changes over age, such as growth and development. In contrast, the demand of ageing as having a deleterious effect on the individual is very often postulated [162]. Ageing itself however, has different forms and definitions:

- **Chronological ageing** is measured in years from a certain starting point, e.g. birth of an individual [3]
- **Social ageing** is the social maturity of an individual to fulfill social or cultural expectations, including the individual’s changing roles in society [71]
- **Emotional ageing** is the change in perception of emotions affecting an individual [145]
- **Biological ageing** is the progressive loss of function needed to meet physiological demands [3]

The latter form mentioned is identical with the definition of senescence, and is the term addressed within this work.

Ageing has always been an interesting subject to mankind - how ageing has evolved, why ageing occurs and what causes ageing. Over a century ago scientists started developing theories of ageing, which over the past years have been approved and discredited many times and continued on being improved and adapted to new scientific perceptions and knowledge. By now, there are hundreds of theories of ageing and are still increasing in number [101]. Here, I will briefly introduce three major theories that have widely been discussed:

1 Introduction

The free radical theory of ageing, proposed by Harman (1956) [65] supposes that a cellular destruction of lipids, proteins, nucleotides by endogenous reactive oxygen species (ROS) results in an accumulative damage with age. This profound theory has experienced much experimental support and popularity as the accumulation of oxidized cellular parts have been discovered in many species over age [155, 126, 161, 123] and organisms supplemented with radical-scavengers and/or overexpression of antioxidants succeeded in prolonged lifespan [58, 33, 154]. A complement of this theory was specified in the mitochondrial theory of free radicals in ageing [105], which states that ROS from post-mitotic cells damage the mitochondrial genome.

Following the publication of Harman's theory, Williams (1957) proposed [189] the theory of antagonistic pleiotropy. This theory is based on the fact that selection due to reproduction only takes place at younger ages and that genes having a deleterious effect at later ages cannot be selected against. This means that genes beneficial early in life that strengthen reproductive success might have detrimental effects later in life. These adverse effects later in life are not selected against and lead to the deleterious outcome of senescence. One example for a gene displaying antagonistic pleiotropy is the $\epsilon 4$ allele of the apolipoprotein E (APOE) gene that is beneficial at younger ages but enhances the risk of Alzheimer's disease later in life [173, 63]. The tumor suppressor gene p53 when under the influence of other genes was also proposed as an example of antagonistic pleiotropy [177].

The disposable soma theory proposed by Kirkwood (1977) [80] is based on the limited resources available for an organism during its lifespan and its allocation at different life stages of an individual. So if more energy is consumed for reproduction, less energy remains for cellular maintenance and repair required for longevity, or vice versa – a so-called tradeoff between propagation and longevity exists. Integrated into this theory is the evolutionary aspect that the body or soma only needs to be maintained as long as reproduction is completed. However, this theory encounters several problems, one of which is the question that if the maintenance of the individual's body took up so much energy, then why not simply continually feed to obtain energy resources as it is done during growth or pregnancy, which are stages requiring great amounts of energy.

A unified theory, however, that includes all aspects of ageing has not been developed so far [180]. So the question of why we age is still open as well as the other pending questions of how we age and how this process might be slowed down, delayed or be otherwise modulated.

1.1.1 Relevance of ageing studies

Life expectancy for humans has never increased as steeply as during the past century, especially for western nations [179] (Fig 1.1). It is widely agreed that this is a consequence of better hygienic standards, improved medical care, a generally well-balanced nutrition and enhanced welfare all arising from technical and industrial advancements.

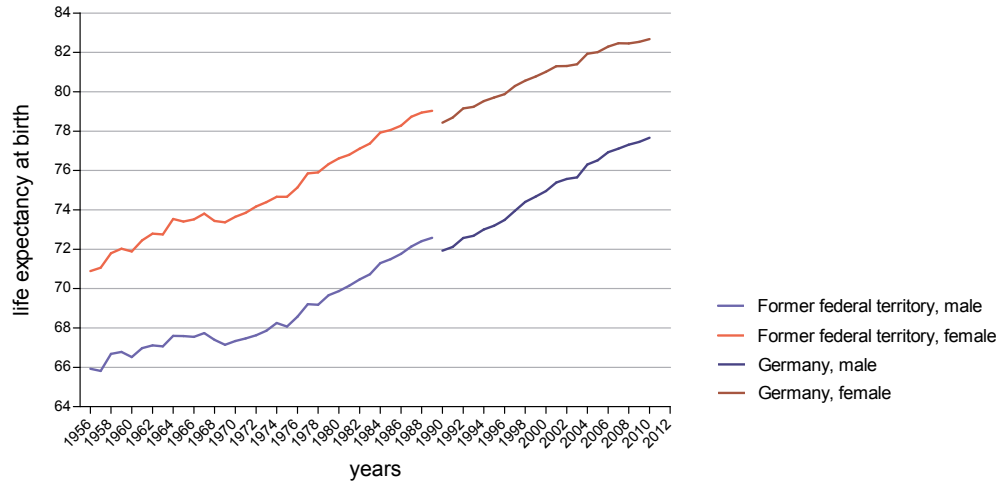


Figure 1.1: Sex specific demographic development in Germany from 1956 to 2010. Copyright: Rostocker Zentrum zur Erforschung des Demografischen Wandels, Datasource: Human Mortality Database.

As the life expectancy steadily increases, so does the incidence of geriatric diseases (Alzheimer's, Parkinson's, cancer, etc.) and the high demand for research on healthy ageing. Studies have shown that about 25 % of the variances in human lifespan can be ascribed to genetic differences [39]. In this relatively new field of science, the group "Forschungsgruppe Gesundes Altern" of our Institute of Clinical Molecular Biology (IKMB) is pursuing this objective and cooperating internationally in genome-wide association studies (GWAS) to investigate the secrets of longevity. In previous studies a number of ageing-relevant or ageing-associated genes have been discovered. One isoform of the apolipoprotein E (APOE) [114], the forkhead box O3A (FOXO3A) [52], as well as a variant of exonuclease 1 (EXO1) [113] have been associated with longevity. Parallel to on-going studies on centenarians (aged >100 years) or even supercentenarians (aged >110 years), comparative studies on different species are also very informative since many cellular mechanisms are well conserved across phylogenetic branches [13]. Investigations on animals, that can reach extraordinary lifespans, such as the >200 year old (expectancy) bowhead whale, >40 year old bats, 140 year old deep sea fishes (e.g. Rockfish), or >30 year old (MLSP) naked mole rat, might show ways to negligible senescence [11, 13, 51]. Such studies aim

to understand the mechanisms of cellular maintenance over a long lifespan without deteriorating effects, *i.e.* healthy ageing to ensure not only the achievement of a long lifespan but also a valuable old age. The organism investigated in the presented work, *Arctica islandica*, reaches a MLSP of more than 400 years [181] without major observed malignancies [124], which naturally makes this animal a very interesting study object in this regard.

1.2 The study object: *Arctica islandica*

Arctica islandica is a marine bivalve mollusk belonging to the superphylum Lophotrochozoan. In the order Veneroida and the family Arctidae the genus *Arctica*, contains only one extant species *Arctica islandica*, which makes it a very phylogenetically isolated species [104, 43]. The class Bivalvia represents the longest-lived metazoan class, and within this class vast differences in MLSP ranging from 1<400 years can be observed [128, 181, 109]. Common names for *A. islandica* are the ocean quahog, and owing to the brownish, black periostracum covering the shell of older individuals, it is also called the black clam or mahogany clam [102]. At younger ages the shell of the ocean quahog has a rather light brown, almost yellow colour [110] and it builds up additional layers of aragonite each year [147]. Like all marine clams *A. islandica* is a filter feeder nourishing on phytoplankton that is taken up through the siphon and filtered by the gills. It is oogamous and reproduces sexually by spawning during warmer summer months, followed by the development of veliger larvae that finally sink to the bottom and grow into clams [118]. *A. islandica* matures at the age of 10-12 years [169] and hermaphrodites have not been observed even though very old (43 years) individuals of indeterminate sex were found [170]. *A. islandica* usually buries into sandy or grained soil and stays beneath the surface for 1-7 days [174, 117]. When cut off from the environmental oxygen supply, ocean quahogs reduce their metabolism and undergo metabolic rate depression (MRD) [168]. Philipp & Abele [128] hypothesized that this energy-saving behavior also reduces the production of disruptive reactive oxygen species (ROS) that emerge from respiration and together with a reduced metabolism might lead to a life-extending effect.

A. islandica (Fig 1.2) is a boreal species distributed across the North Atlantic along continental shelves. Its southern distribution boundary has been reported to be from Cape Hatteras, North Carolina, USA, at the western Atlantic coast reaching up north to Newfoundland and Labrador, Canada [102, 117]. The species is also abundant along the coasts of Iceland, the Faroe Islands of Denmark, and on the European continental shelf up to the North cape in Norway [102]. It is commercially



Figure 1.2: *Arctica islandica*

harvested along the coasts of the USA, Canada and Iceland but not in other parts of the world [102, 192]. *A. islandica* can live in waters with temperatures ranging from 0°C to 16°C and is commonly found alive in water depths between 10 and 280 m, and occasionally as deep as 500 m [95, 117]. Studies confirmed that although all populations of *A. islandica* are widely distributed, they clearly belong to one species [43] and that genetic variability within population seems to be higher than variability between populations (personal communication with D. Abele and C. Held at the Alfred-Wegener Institute, Bremerhaven, who performed 16S rDNA and Cytb investigations on European *A. islandica* populations). The oldest individual of this species reaching a MLSP of >400 years has been recovered Northeast of Iceland [181], whereas in other regions, such as in the Baltic Sea the MLSP is less than 50 years [19]. In the Baltic Sea, *A. islandica* seems to be driven to the edge of its tolerable habitat since it has to endure the greatest fluctuations of different environmental factors, such as salinity, temperature and oxygen availability [16]. The ocean quahog presumably suffers more stress from low salinity values in the Baltic Sea [18] than in Northeast of Iceland where there is more stable conditions of low temperature, higher salinity and oxygen-rich water [16].

1.2.1 *A. islandica* as model organism for ageing

There are several requirements that classic model organisms should meet. The organisms should (i) have short generation times so that multiple generations can be studied, (ii) large numbers should be easily kept and maintained in the laboratory, (iii) have its genome available and (iv) be easily manipulated genetically [24, 139, 176]. Common classic model organisms that meet these requirements are the nematode worm *Caenorhabditis elegans*, the baker's yeast *Saccharomyces cerevisiae*, and the fruitfly *Drosophila melanogaster*. The house mouse *Mus musculus* usually serves as a popular vertebrate model organism for studies on species more closer related to

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humans [24]. However, for certain model organisms, for example, flies and worms that belong to the superphylum Ecdysozoa, many genes vanished until the separation from the common ancestor with humans, making it a major drawback for comparative studies [12]. It is also known that the lifespan of model organisms kept in the laboratory is affected by the laboratory environment [122]. This can result in lifespan not only adapted to routine feeding conditions but also to artificial life conditions free from pathogens and natural enemies [122]. Additionally, uncontrolled changes like mutations due to inbreeding and noise and smell, that are hard to reconstruct across different laboratories, affect the model organism lifespan, the outcome of the experiment [122]. For these reasons, taking the studied organism directly from their natural habitat can circumvent these laboratory issues. In contrast to mice and other animals captured from the wild, the age of *A. islandica* can be exactly determined by the count of age rings each individual accumulates in its shell similar to those observed in the trunks of trees [192, 138, 28].

Since the question on how an individual can reach such an extraordinarily long lifespan remains uninvestigated so far, it seems only natural to study an organism with an outstanding MLSP such as *A. islandica* (>400 years in Iceland population) instead of another short-lived species. Mechanisms for longevity should have evolved in a long-lived species rather than in the short-lived classic model organisms studied so far. Besides its long lifespan, *A. islandica* also grows and reproduces throughout its long life [138, 169] indicating that selection does not only happen during a certain stage of its lifetime but during the complete lifespan after maturation. The problem facing the antagonistic pleiotropy theory of ageing in that some genes have a beneficial effect earlier in life but are detrimental later on is therefore negligible when working with the bivalve mollusk. Needless to say, experiments on living quahogs over generations cannot be acquired due to the great lifespan of the animals even though the husbandry of the animals in aquaria have been accomplished over years several times [160] (personal observation). Hence, cross-sectional analyses of sampled populations of *A. islandica* from the wild with immensely differing MLSPs from the Baltic Sea (MLSP 40 years) and Iceland (MLSP >400 years) that give information on the ageing process and mechanism of the bivalve have to be performed. The investigation of several parameters, especially the population specific molecular mechanisms described in the following, should shed then light on the ocean quahog's secret to longevity.

1.3 Molecular biology background

Every organism changes over time whereby it undergoes physical, mental and molecular changes. Molecular changes in the genome of an organism can occur due to spontaneous mutations that are neglected or overlooked by the DNA repair machinery (see 1.3.2). Genomic changes can also occur due to extrinsic environmental influences that can cause mutations in the genetic code and by other chemical modifications to the genetic material, such as methylation of histones, chromatin modifications or regulation of noncoding RNAs (referred to as epigenetics) [25]. Affected genes can be regulated by epigenetics so that gene expression changes in an organism over age. For instance, genes important for the development of an organism are highly expressed at early stages in life compared to total silencing or down-regulation of these genes after maturation at later stages. The resulting biochemical changes in cells include the decline of certain molecules over time simply by wear and tear, neglected maintenance and the accumulation of waste material that cannot be disposed of all leading to malfunctioning mechanisms. Biochemical parameters investigated in this study are outlined below and a description of the molecular investigations follows in 1.3.3.

1.3.1 What are ageing markers?

Every living system transforms energy to heat, motion, growth, or reproduction. Energy transformation is, however, never 100 % efficient since waste byproducts damage other cellular compartments or accumulate if they cannot be repaired, degraded or be disposed of. Non-degradable waste products that may also arise from external damage, such as through ultraviolet (UV) light, ionizing radiation (IR), chemicals, toxins and pollutants [98], accumulate over time in an organism. The damage can be measured as so-called ageing markers or biomarkers of ageing [15], e.g. oxidation of proteins, lipids and nucleotides. Other ageing-markers are characterized by their depletion or diminished function over age, for instance, protein stability, telomere length and telomerase activity. The oxidation of cellular components (such as proteins, lipids, and nucleotides) can easily be explained by the attack of reactive-oxygen-species (ROS) internally generated by the electron transport chain at the membranes of mitochondria during the process of respiration. Aggressive ROS arising from this process are arrested by oxidative-stress defense systems through antioxidants such as vitamin A, C, and E, and glutathione, and by ROS-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), and various peroxidases (e.g. GPx).

1.3.1.1 Protein oxidation

Reactive-oxygen-species can lead to the oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation [22]. Protein oxidation can be quantified through protein carbonyls that are formed by direct oxidation of the amino acids lysine, arginine, proline, and threonine residues [22]. Quantification is achieved by the reversible coupling of the fluorescein-5-thiosemicarbazide (FTC) dye and detection of emitted fluorescence [36]. To date, protein carbonyl content is the most widely used marker of oxidative modification of proteins and it has been demonstrated that oxidative damage to proteins correlates well with ageing and the severity of some diseases [38]. The accumulation of protein carbonyls over age has been shown in several vertebrate and invertebrate organisms [155] amongst which are clams including *A. islandica* [126, 161]. A distinct protein carbonyl content could be determined in several studies comparing longer-lived to shorter-lived clams and scallops [126, 125] suggesting a difference in this ageing-marker between the long-lived Iceland and the short-lived Baltic Sea populations of *A. islandica* with vast difference in MLSP.

1.3.1.2 Lipid peroxidation

Isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids and are accurate markers of lipid peroxidation [106]. F₂-isoprostanes (F₂-IsoPs) quantification is conducted by gas chromatography separation and enhanced mass spectrometry [107]. Measurements of F₂-IsoPs are indicative for oxidative stress during pathogenesis of human diseases [106]. However, correlation of increased lipid peroxidation with age has been controversially discussed, and seems to be species-, strain-, sex-, and tissue-specific [136]. In contrast, reduced lipid peroxidation in long-lived bat species compared to shorter-lived mice has been observed [188]. Additionally, accumulative lipid peroxidation over age could be observed in the blue mussel, *Mytilus edulis*, [163] and a significant increase of lipofuscin, the end product of lipid peroxidation, over age was detected in the long-lived population of *A. islandica* from Iceland [161]. Thus, a comparison of lipid peroxidation between the long-lived and short-lived populations of *A. islandica* studied in this work is of high interest.

1.3.1.3 Nucleotide oxidation

The oxidation of nucleotides through ROS results in the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) in DNA and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) in RNA, and can be quantified as the ratio of oxidized to non-oxidized deoxyguanosine or guanosine by high-performance liquid chromatography coupled to electrochemical detection (HPLC-ECD) [69]. An estimate of 100-500 8-oxodGuo lesions are produced in a human cell per day [92]. Unrepaired DNA damage due to oxidative stress greatly elevates the risk for cancer and other diseases [68]. In contrast to other compounds damaged by ROS, such as proteins and lipids that can be replaced, lesions to DNA result in major damage to nucleotides that remain as part of the genome are transmitted to daughter cells if not removed through apoptosis [90]. An increase of oxidized nuclear DNA as well as mitochondrial DNA over age was measured in mice and other rodents [62] but to date has not been measured in bivalves. Hence, an investigation of this ageing-parameter in the bivalve *A. islandica* may give information on the DNA repair capacity of this long-lived organism.

1.3.1.4 Protein stability

Pérez *et al.* [123] hypothesize that besides the accumulation of oxidized proteins, the maintenance of protein structural stability also plays an important role in ageing. Toxic protein oligomers or aggregates commonly observed in neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington diseases, may be accelerated due to loss of the functional structure of proteins [75]. It has also been shown that proteins in long-lived bats displayed enhanced stability when exposed to urea-induced protein unfolding compared to shorter-lived species of mice [142]. Additionally, the long-lived naked mole rat exhibited a higher protein stability compared to the shorter-lived related mice, which also showed increasing protein instability with increasing age [123]. Similar to previous studies, this study measured protein stability using the preferential binding of the hydrophobic fluorescent molecular probe, BisANS (4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid), to partially folded intermediate proteins and further relation to total protein content gives information on the total protein stability [152].

1.3.1.5 Telomere length and telomerase activity

Telomeres are DNA-protein structures found at the ends of chromosomes, that protect the DNA against degradation, recombination and random fusion by cellular DNA repair systems [35]. Telomeres shorten with each cell cycle due to the DNA end-replication problem [183] of DNA polymerase (refer to 1.3.2) being unable to replicate the terminal 3' end of the DNA strand leading to incomplete replication and reduction of the 5' end by 100-200 bp per each replication process [64, 88]. When the chromosomal ends become too short reaching the so-called Hayflick limit [66], cells may enter cellular senescence [35]. Truncated telomeres have been observed in human fibroblasts over age [64]. This shortening of telomeres is considered as one responsible factor for the irreversible loss of proliferative capacity of somatic cells with age. The telomere structure with the repetitive sequence of 5'-TTAGGG-3' is highly conserved among vertebrates [103] as well as among invertebrates [172]. This repetitive sequence could also be identified in genomic sequences of *A. islandica* (personal communication with C. Held, Alfred-Wegener-Institute Bremerhaven).

The shortening of telomeres can be prevented by ectopic expression of telomerase. This enzyme can stabilize or even elongate telomeres, regenerate the original length and theoretically lead to unlimited proliferative potential [10]. Expression of telomerase can be observed in germ cells [196], stem cells [97], in actively proliferating transit cells [30], and has been detected in >85 % of various types of human cancer cells [79, 83]. Telomeric repeats of the highly conserved sequence have been detected and lengths measured in several tissues of scallops *Argopecten irradians irradians*, *Argopecten purpuratus* and *Euvola ziczac* [48, 121] and other marine invertebrates, such as the wedgeshell clam *Donax trunculus*, mussel *Mytilus galloprovincialis*, Pacific oyster *Crassostrea gigas*, scleractinian corals *Madracis auretenra* and *Madracis decactis*, red sea urchin *Strongylocentrotus franciscanus*, sea cucumber *Holothuria tubulosa*, and colonial ascidian *Diplosoma listerianum* [54, 60, 132, 153, 200]. Additionally, a decrease in telomere length with age in gill tissue of scallops was observed [48]. Thus, investigating telomere length and telomerase expression over age in the short- and long-lived populations of *A. islandica* could help to assess the role of telomere maintenance for longevity of this organism.

1.3.2 DNA replication and repair

During growth of an organism and as a consequence of wear and tear, tissue and cells are built up and replaced. The cell must copy its genetic makeup before it

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can divide to produce new cells. In somatic cells this replication process happens during mitosis and in gametic cells this process, which also includes recombination of genetic material happens during meiosis. Since each cell contains the complete genetic makeup of an organism, the genetic material needs to be replicated by a complex replication machinery, in which the DNA polymerase enzyme plays the main part, before new cells may emerge. Even during the replication of a human genome, which contains 3.2 billion base pairs and only $\sim 2\%$ are coding sequences, less than one error for every billion base pairs occurs [87, 99]. However, since the genome constantly needs to be replicated for a whole lifespan, errors in the genetic code would be transmitted to each daughter cell and accumulate quickly if not repaired. Hence, a complex DNA repair machinery has evolved to compensate for damage to DNA. Nevertheless, this machinery may not keep up with continuous lesions to DNA so that they may accumulate in an organism over age. Next to the concomitant proofreading function of some polymerases that can excise erroneously incorporated base pairs and replace them with the correct base, several repair mechanisms exist for wrong base pairings and other damage to the DNA. A selection of DNA repair mechanisms that are covered in this work is introduced here.

The incorporation of base-pair mismatches into the synthesized complementary DNA strand that results in loops and bubbles are recognized and repaired by the DNA mismatch repair (MMR) system [143]. This repair system not only plays a role during DNA replication, but also during the processing of recombination intermediates, the regulation of recombination events, nucleotide excision repair (NER), and it also participates in a cell cycle checkpoint control system [84]. However, most DNA damage does not emerge from DNA replication but rather as a consequence of extrinsic and intrinsic disruptive influences resulting in mismatches as well as single-strand DNA breaks (SSB), double-strand breaks (DSB), chemical modifications of bases or sugars, and inter- or intrastrand cross-links [34, 44, 67, 86]. The major source of endogenous DNA damage is ROS that are generated during normal cellular metabolism [23, 92]. Oxidatively damaged bases, such as 8-oxodG (see 1.3.1.3), are primarily repaired by base excision repair (BER) pathways that involve the core proteins APE1, POL β , DNA ligase 1, FEN1, and XRCC1 [98]. Supporting evidence that the functionality of BER and its components play a role in ageing and ageing-relevant diseases, such as cancer, is reviewed in Maynard *et al.* [98]. Repair of DSBs in most metazoans is performed through Non-homologous end-joining (NHEJ) [90]. This mechanism relies on the re-synthesis of damaged DNA segments after hybridization of microhomologies that are often present at the single-stranded overhangs of DSBs. Perfect compatibility of these homologies results in accurate repair which is rare and, more commonly, the microhomologies are not perfect

resulting in imprecise repair that leads to loss of nucleotides [90]. Premature ageing when knocking down core proteins (e.g. Ku) in the NHEJ pathway has been observed in mice [90]. The human premature ageing disorder, Werner Syndrome, can be ascribed to the functional loss of WRN, a helicase that plays a role in NHEJ as well as homologous recombination (HR) [37]. Homologous recombination relies on the mechanism of DNA synthesis of an excised section by hybridization of the defect DNA to the second homologous intact chromosome. During this process, there is no loss of nucleotides and hence, it is much more accurate, but can only be accomplished during diploid stages of the cell cycle. Karanjawala & Lieber [90] suggest that causes of senescence probably result from nuclear DNA lesions since main premature-ageing syndromes in humans involve nuclear proteins. The authors also state that lesions to nuclear DNA is much more severe compared to damage in mitochondrial DNA since the latter is present in the cells at much higher copy numbers. Since DNA is prone to the attack of intrinsic- and extrinsic-originating ROS, the study of the two *A. islandica* populations with such distinctly different environments (see 1.2) and such vast differences in MLSP shall elucidate population-specific evolved defense or repair mechanisms.

1.3.3 What is a transcriptome?

The above mentioned ageing-markers are all build up and mainly regulated by the molecular machinery that determines types and amounts of proteins produced, and helps arranging lipids and regulating nucleotides. It is herewith necessary to additionally investigate types and levels of gene transcripts as described in the following to fully understand the process of ageing.

The genome of an organism reflecting the total genetic information in the DNA – all the genes, coding, and non-coding, as well as non-transcribed – is roughly equivalent in each cell. In contrast, the transcriptome, which represents all transcripts captured in RNA molecules, are variable depending on cellular state, location and type, and on external conditions. These RNA molecules can be roughly divided into groups: the messenger mRNA make up approx. 4 % of total RNA in a cell and carry the information for amino acid sequence in proteins, transfer tRNAs carry the amino acids to the site of protein synthesis in the ribosomes, which are constituted of ribosomal rRNAs that make up approx. 80 % of total RNA [29]. A transcriptome usually encompasses all cellular transcripts (mRNAs), non-coding tRNAs and rRNAs, and several other RNA molecules, such as small nuclear snRNA, small nucleolar snoRNA, micro miRNA, etc. The composition of the transcriptome can vary according to experimental sampling. If a pre-selection for mRNAs is performed, the sampled

transcriptome then only represents the genes expressed at the time of sampling. The transcriptome differs also between life-stages of an organism, hence, over age. Genes expressed during development, or early stages of embryology, are often silenced (e.g. telomerase in humans) since not needed or even detrimental during adult life-stages [53]. In addition to descriptive information on which genes are expressed at an investigated state, a transcriptome also reflects quantitative information on gene expression levels. Investigating gene expression over age in the long-lived Iceland and the short-lived Baltic Sea population may, hence, point towards important gene regulation over age and also reveal distinct expression behaviour between the two populations.

Thus, a transcriptome can give information on the conditions the organism was undergoing by looking at the expressed genes and their level of expression, and conversely, given experimental setup conditions show which genes are expressed at which level under these circumstances. The approach taken in this study refers to the latter mentioned with sampling of different aged *A. islandica* individuals from long- and short-lived populations and looking for genes differently expressed along different ages and between the two populations. To depict all transcripts present at time of sampling, the sampled RNAs are converted to copy cDNAs (by reverse transcriptase) prior to sequencing since the sequencing methods involve the amplification of input DNA. The next-generation sequencing (NGS) technique used in this work will briefly be introduced in the following section.

1.3.3.1 Next-generation sequencing

The Gold-Standard technique to determine nucleotides sequences is the chain termination method introduced by Frederick Sanger *et al.* (1977) [144]. This method relies on the termination of nucleotide strand synthesis by polymerases – enzymes responsible for DNA replication during the process of cell division (see 1.3.2) – due to the incorporation of dideoxynucleotides (ddNTPs) missing the 3' hydroxyl group required for the formation of a phosphodiester bond during elongation of a nucleotide strand. Although this technology is qualitatively very reliable generating sequence reads up to 1,000 base pairs, it is not suitable for high throughput usage needed for genome or transcriptome sequencing due to its expensive chemicals. New technologies producing tremendous amounts of sequences at low costs, such as Illumina sequencing, SOLiD sequencing and pyrosequencing, have thus been developed and are referred to as next-generation sequencing (NGS).

The method used in this work is pyrosequencing which is based on sequencing by

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synthesis, and was first invented by Pyrosequencing AB in Sweden. It was further licensed to 454 Life Technologies who developed an array-based pyrosequencing technology. The technique relies on the detection of pyrophosphate (PP_i) released upon nucleotide incorporation. In this procedure, DNA is sheared by nebulization, immobilized on beads and amplified by emulsion PCR (Fig 1.3A-B). The resulting DNA library on the beads is distributed onto a picotiter plate (PTP) comprising of 1.6 million single wells, so that each well contains one bead (Fig 1.3C).

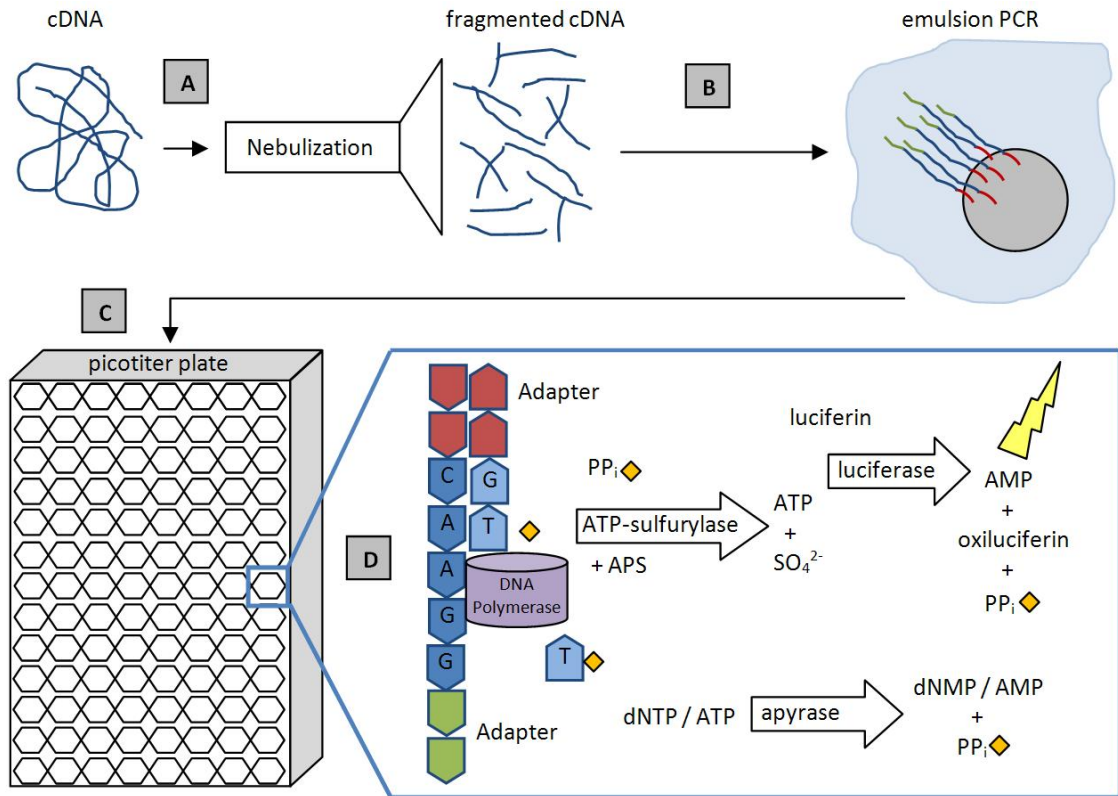


Figure 1.3: Preparatory steps and pyrosequencing reaction. Total cDNA is fragmented by nebulization (mechanic shearing of cDNA by forcing it through a small hole) (A). Fragmented cDNA is further provided with two different adapters (shown in green and red) for each end of the fragment (procedure not shown). Single fragments are attached onto beads, and amplified during an emulsion-PCR (B). Each bead covered with clonally amplified sequences is distributed into one well of a picotiter plate (C). Synthesis of the complementary strand on the bead takes place with the addition of DNA polymerase that elongates the synthesized strand by one nucleotide at a time and releases PP_i (orange diamond). The added ATP -sulfurylase and APS provide ATP for the conversion of luciferin into oxyluciferin generating light (depicted by yellow lightning) by luciferase. Remaining $dNTP$ s and ATP s are degraded into $dNMP$ s and $dAMP$ s by apyrase before the addition of reagents for the next reaction cycle (D). Dimensions are not to scale.

The sequencing output after loading one-half of a PTP is about 500,000 reads.

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454 pyrosequencing results in an average sequence length of 400-500 bp. All reads represent fractions of transcripts that further need to be aligned and assembled into contigs. This is generally facilitated by mapping the reads onto a known reference genome. Transcriptomes of organisms without a reference can be mapped onto genomes or transcriptomes of closely related organisms or a *de novo* assembly must be performed. Other NGS techniques (e.g. Illumina, SOLiD) yield reads with ≤ 100 bp length which, for a *de novo* assembly, is too short to result in sufficient overlaps to correctly assemble the reads into contigs. A *de novo* assembly is made even more complex since some genes can result in various protein products (protein isoforms) due to alternative splicing of introns. Additionally, since many proteins are built up by recurring domains the attribution of single domains into the wrong contigs may lead to a false expression level assigned to that contig or area of a contig. The amount of PTPs or sections that are sequenced is selected depending on the genome size of the investigated organism and the desired average base pair coverage. Synthesis of the complementary strand of DNA library fragments takes place in each PTP well by the addition of a DNA polymerase, ATP sulfurylase, luciferase, apyrase and the substrates adenosine 5' phosphosulfate (APS) and luciferin. Sequential addition of one kind of nucleotide at a time (A, T, G, or C) by DNA polymerase releases a pyrophosphate molecule which together with substrate APS is converted to sulfate and one molecule of ATP by ATP sulfurylase. A chemiluminescent signal is released through the ATP-triggered conversion of luciferin to oxyluciferin, AMP and PP_i by the luciferase enzyme. The light produced by conversion of ATP is proportional to the number of specific nucleotides added to the synthesized strand and detected by a camera. Non-incorporated dNTPs and remaining ATPs are degraded by apyrase (Fig 1.3D). Since the released signal does not distinguish between the different nucleotides, the identity of the different nucleotides in each reaction must be known. Since ATP serves as substrate for luciferase and is also one of the nucleotides needed for strand elongation, a modified deoxyadenosine alpha-thio triphosphate (dATP α S) that serves as substrate for DNA polymerase, but not for luciferin, is added for strand elongation. The sequence output of this technology with 400-600 Mb is significantly less compared to other NGS methods mentioned previously resulting in a lower average base coverage when using pyrosequencing [45, 187, 96].

1.3.3.2 quantitative realtime polymerase chain reaction

As mentioned above, a quantitative determination of gene expression can be achieved by investigating a transcriptome. However, the resolution of gene expression over age from a transcriptome is not very distinct when only looking at few individual

samples at separate reflected time points. A more progressive gene expression over age can be obtained when looking at a higher number of individuals over a wide age range. This approach was undertaken using quantitative realtime polymerase chain reaction (qRT-PCR) in order to investigate the expression of selected genes over age in the two *A. islandica* populations.

In qRT-PCR the DNA polymerase is used to obtain a rapid exponential amplification of a target DNA section. Quantification of the initial amount of target DNA can be achieved by a fluorescent dye (e.g. SYBR green) incorporated in primers targeting the DNA section of interest. Each copied section serves as a template for the next reaction yielding an exponential amplification of target, commonly achieved in 25-40 PCR cycles. From the fluorescence measured after completion of each cycle, the amount of initial target can be deduced either by the simultaneous measurement of a known standard or comparison with a reference target, such as a housekeeper gene. A relative quantification of a gene of interest to a housekeeper gene can ultimately be obtained.

Quantitative realtime PCR has previously been used to validate microarray data and find distinct gene expression in different old individuals of the sea urchin *Strongylocentrotus purpuratus* [93]. Due to previously mentioned assembly issues that might affect expression levels depicted in a transcriptome, it is necessary to validate and investigate detailed gene expression of genes of interest over age in the long- and short-lived *A. islandica* populations.

1.4 Aims

It is generally agreed that there are no single genes responsible for senescence or genes that could be modified to reach Methuselah's age. This project sought to study the underlying mechanisms and genes involved in ageing by answering the following questions:

- i. Can we find a significantly lower level of cellular damage in the longer-lived population that could contribute to the individual's ability to maintain its soft body for such a long time? There are several damage parameters that accumulate in an organism and others that decline with age. A selection of these ageing markers measured in different old individuals of the short-lived Baltic Sea and long-lived Iceland *A. islandica* populations may allow comparison of the ability of cellular maintenance between the two populations.

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- ii. Do we find changes in ageing related transcriptional profiles that differ between the two populations of *A. islandica*? In other words, do both populations respond differently regarding their gene expression throughout their ageing process, and might this difference be revealed by dissimilar up- or downregulation of specific and unknown genes. The complex compilation of several transcriptomes representing the gene expression of both populations along different ages serves as a tool for transcriptional profiling analyses.
- iii. Can we identify ageing-relevant genes and pathways that also play a role in the ageing process in other organisms *i.e.* humans? Here, different approaches were undertaken to best answer this question. First, from the identification of annotated ageing- or repair-associated genes through the transcriptome, the detailed expression of selected target genes could be analyzed via quantitative realtime PCR (qRT-PCR). Second, analyses for the enrichment of pathways and GO-terms more frequently occurring in a certain age group, population, or tissue were undertaken in the transcriptome.

Through these questions I aim to understand how one population of the same species can live ten-fold longer than the other by looking at which mechanisms allow for maintenance of tremendous long life and which relevant and/or new genes and pathways, not yet discovered, enable for this immense lifespan of more than 400 years.

2 Materials and Methods

2.1 Characterization of animals

In this work, the ageing characteristics of two populations of *Arctica islandica* (L.), a short-lived population in the Baltic Sea (MLSP \sim 40 years) and a very long-lived population close to Iceland (MLSP $>$ 400 years), are studied. The procedures and methods used to accomplish this task are described in the following sections.

2.1.1 Sampling

In the Baltic Sea 160 *A. islandica* individuals were sampled at the station "Süderfahrt" (54°32.6N 10°42.1E) in February 2010. In March of the same year, 179 Icelandic animals were collected from Northeast of Iceland (66°01.5N 14°50.9W). Both populations were recovered from water depth of 20 m with a hydraulic dredge and kept under stable laboratory conditions (with fresh water flow through, approx. 5°C water temperature, approx. 35 ppt salinity in Iceland, and 20-25 ppt in the Baltic Sea) at the GEOMAR Helmholtz Centre for Ocean Research (Kiel, Germany) for the Baltic Sea animals and the Suðurnes University Research Centre (Sandgerði, Iceland) for the Icelandic animals. After 5 days of recovery from sampling stress, the animals were dissected and various tissues were sampled. The gill, mantle, foot, adductor muscle, heart, and digestive gland were shock-frozen in liquid nitrogen and stored at -80°C until further analysis. For the sex determination, gonad tissue was sampled, fixed in Baker's Solution (4 % formaldehyde, 2 % w/v calcium acetate) and stored at 4°C for at least 10 days.

2.1.2 Morphometrics

Since the difference in morphometrics of both investigated *A. islandica* populations is quite obvious, various size parameters (Fig 2.1) of the sampled animals were taken

as described in the following. Prior to the dissection, all animals were individually weighed to determine whole animal weight. Shells of all individuals were cleaned, dried, and length, width, and height (line of strongest growth = LSG) were measured with calipers to the nearest mm (Fig 2.1). Dried shells were weighed to the nearest 0.01 g. Soft body weight was calculated by subtracting dry shell weight from whole animal weight.

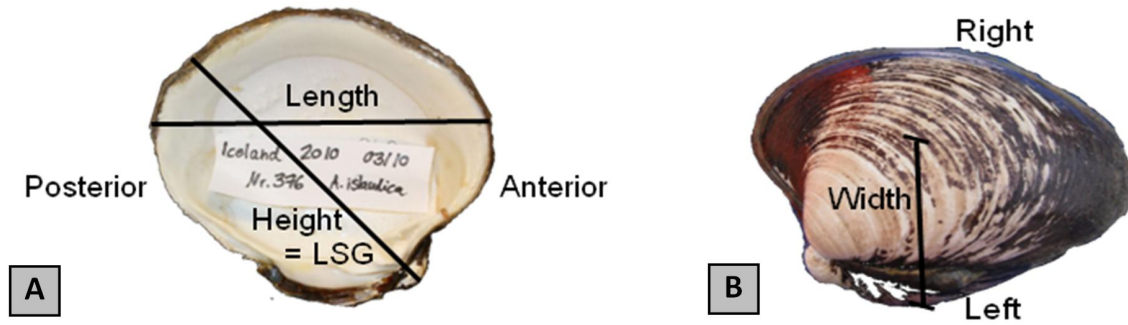


Figure 2.1: Morphometrics of an *A. islandica* shell. Length and height (LSG) with posterior and anterior sides (A) and width with left and right half of the shell indicated (B).

2.1.3 Sex determination

Sexes of *A. islandica* can be determined via histological sections through the gonads [170]. The fixed gonad tissue was dehydrated and embedded in paraffin. Serial sections (4.5 μm) were made with a Leica Microtome RM 2255 (Leica Biosystems, Wetzlar, Germany) and dried on a microscope slide at 37°C over night.

Histological stains were performed as follows: slide sections were placed into xylol substitute (Roti-Histol) for 10 min, gently shaken in 3x100 % EtOH, 2x96 % EtOH, 1x70 % EtOH for 30 s in each. Slide samples were heavily shaken in dH₂O, followed by gentle shaking in replaced dH₂O for 5 min to remove the paraffin. Slides were subsequently washed 4-5 min in haematoxylin, then placed into H₂O and rinsed under running water for 10-15 min. Slides were then washed in 0.5 % eosin solution for 6 min and briefly rinsed in H₂O. For embedding, slides were quickly placed in increasing EtOH baths of: 1x70 % EtOH, 3x96 % EtOH, 2x100 % EtOH and then placed into 3x100 % EtOH, 3x xylol substitute for 30 s each. One drop of Roti-Histokitt was added onto the microscope slide and carefully covered with a coverslip. Stains were then air-dried and sex was determined under a Medicus HF:Hellfeld Microscope (Hund, Wetzlar, Germany) at a 10-fold magnification (Fig 2.2).

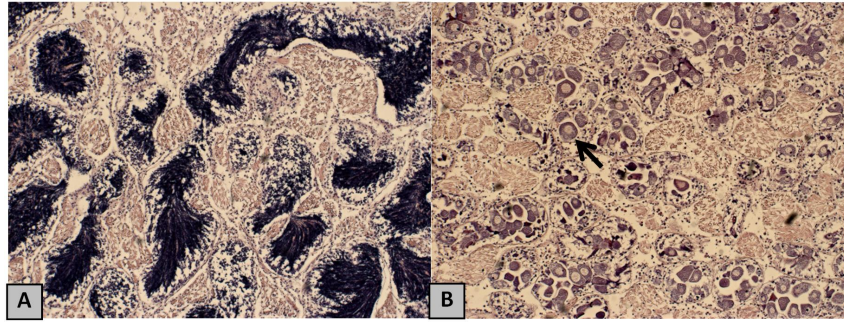


Figure 2.2: HE-stains of male (A) and female (B) gonads of *A. islandica*. Clusters of sperm cells can be seen in (A) as dark areas and single egg cells can be detected in (B) (see arrow). Pictures were taken under a Zeiss Imager Z.1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and AxioCam HRc with the AxioVision software.

2.1.4 Age determination

A. islandica lay down annual growth rings in their shell [146, 192] from which the animals' individual ages can be deduced. The preparation and processing of the shells were generally performed as described by Strahl *et al.* [161] and Begum [19]. The right half of all shells was freed from organic remains with 13 % NaOCl and the line of strongest growth (LSG) was embedded in two component liquid metal glue (Toolkraft, Georgensgmünd, Germany) (Fig 2.3A). A 1 cm wedge comprising the LSG was cut out with a diamond blade using an FKS/E bench saw (Proxxon, Niersbach, Germany). The wedge was adhered onto a wooden support in order to clamp it into an IsoMet low-speed saw (Buehler, Lake Bluff, IL, USA) to perform dissections along the LSG (Fig 2.3B). The shell surface was sanded using P400, P800, P1200 grits (P2500 and P4000 grits were used whenever necessary) (Fig 2.3C) and subsequently polished using 1.0 micron Buehler Micropolish II (Buehler, Düsseldorf, Germany). The age was determined by counting age rings under a 2.5 magnification lens of a Zeiss Imager Z.1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and AxioCam HRc with the AxioVision software (Fig 2.3D).

2.2 Ageing markers

Several biological markers are indicative for ageing. The following markers were investigated according to age and to differences between the long- and short-lived populations of *A. islandica*.

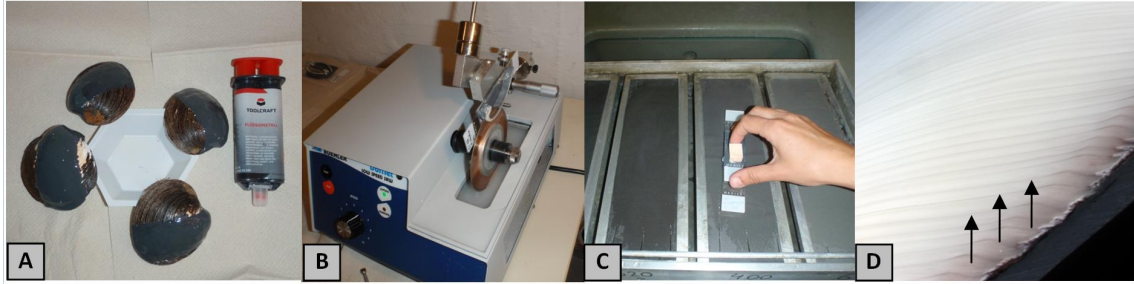


Figure 2.3: Processing of *A. islandica* shells for age determination: Shells embedded in liquid metal glue (A). Cutting along the LSG with a low-speed saw (B). Sanding of the shells (C). Microscope image of age rings (arrows) within the shell (D).

2.2.1 Measurement of protein carbonyls

Soluble and insoluble protein carbonyls of 15 Baltic Sea (age range 11-35 years) and 25 Iceland animals (age range 11-226 years) were stained with fluorescein-5-thiosemicarbazide (FTC) in order to quantify the labeled oxidised protein relative to total protein content (F/C ratio) according to Chadhuri *et al.* [36]. First, gill tissue (150 – 200 mg) was homogenized in 350 μ l Potassium Phosphate buffer (20 mM KPO_4 , 0.5 mM MgCl_2 , 1 mM EDTA, pH 6) with protease inhibitor cocktail (Roche, Mannheim, Germany) and Zirconium Silicate Beads using a Retsch MM 301 homogenizer (2 min with 30/s). The lysate was ultracentrifuged in a Beckmann TL-100 ultracentrifuge for 1 hour at 100,000 $\times g$ at 4°C. The supernatant was transferred into a small reaction tube and kept on ice. The pellet was resuspended in 200 μ l P3 buffer (20 mM KPO_4 , 0.5 mM MgCl_2 , 1 mM EDTA, 2 % SDS, 0.5 % IGEPAL, 0.5 % Sodium Deoxycholate, pH 6) with Roche protease inhibitor cocktail and 1 mM DTT. The resuspended pellet was sonicated for 10 s on ice, and ultracentrifuged at 100,000 $\times g$ for 10 min at RT. The protein concentration of both supernatants was measured using Pierce BCA assay (Thermo Scientific, Rockford, USA). For measuring protein carbonyls, a 500 μ l reaction mix of 0.3 M Guanidine in KPO_4 buffer, 1 mM FTC label and 500 μ g of protein was prepared. The total mix was incubated at 37°C for 2 hours. After incubation, proteins were precipitated by the addition of 500 μ l of 20 % chilled trichloroacetic acid (TCA) in KPO_4 buffer and incubation on ice for 15 min. After centrifugation at 16,000 $\times g$ at 4°C for 15 min, the supernatant was removed. Pellets were washed 6 times in ethanol/ethyl acetate (1:1) by repeated disruption with a spatula followed by centrifugation at 16,000 $\times g$ at 4°C for 15 min. Tubes were kept on ice at all times to avoid protein dissolving. After the last wash, excess ethanol/ethyl acetate was removed and protein pellets were dissolved in 50 μ l of 8 M Urea in KPO_4 buffer and incubated at 37°C for 30 min. Dissolved pellets were sonicated for 20 s and then centrifuged at 16,000 $\times g$ at RT for

2 min. This step was repeated to increase protein concentration. After measuring the protein content by BCA Assay 6.66 μg protein with 1x reducing loading buffer were loaded onto an SDS gel and run at 150 V. The image of fluorescent protein was captured with a Typhoon 9400 (GE Healthcare, Munich, Germany) using an excitation wavelength of 488 nm and an emission filter at 520 nm with a PMT value of 460. The gel was then washed 3 times for 5 min with water and stained with Bio-safe coomassie (BioRad) overnight. The gel was washed with water until all residual stain has disappeared before capturing the coomassie image with a visible camera (Molecular Imager ChemiDoc XRS, Bio-Rad, Munich, Germany). The fluorescence and coomassie images were analyzed using ImageJ 1.45S and the data expressed in F/C ratios.

2.2.2 Measurement of lipid peroxidation

In a collaboration with Prof. S. Austad, Director of the Barshop Institute for Longevity & Aging Studies at the UT Health Science Center San Antonio, USA, F₂-isoprostanes (F₂-IsoPs) were measured in gill tissue of 15 Baltic Sea (age range 11-31 years) and 25 Iceland animals (age range 11-226 years) as markers for lipid peroxidation. F₂-IsoPs were determined using a stable isotope dilution method with detection by gas chromatography/negative-ion chemical ionization/mass spectrometry (GC-NICI-MS) as described by Morrow *et al.* [107]. Briefly, 100-200 mg of gill tissue was homogenized in ice-cold Folch solution (chloroform/methanol 2:1) containing 5 mg/100 ml butylated hydroxytoluene (BHT). Lipids were then extracted and chemically hydrolyzed with 15 % Potassium Hydroxide. After acidification with HCl, a stable isotope, 8-*iso*-prostaglandin F_{2 α} -d₄ internal standard was added. Following extraction using C-18 and silica Sep-Pac cartridges, the eluted compounds were dried under nitrogen (N₂) then converted to pentafluorobenzyl esters, and purified by thin-layer chromatography (TLC). The purified F₂-isoprostanes were derivatized to trimethylsilyl ether derivatives and then dissolved in undecane for quantification by GC/MS. Negative ion chemical ionization MS was performed by Agilent 6890 GC and Model 5975 MSD instruments with selected ions monitored for [²H₄]15-F_{2 α} -IsoP internal standard (m/z 573) and F₂-IsoPs (m/z 569).

2.2.3 Measurement of RNA and DNA oxidation

The measurement of oxidated RNA and DNA was done as commissional work by the laboratory of Prof. C. Leeuwenburg together with Prof. S. Wohlgemuth, University

of Florida, USA. In this procedure, oxidized RNA and DNA of 21 Baltic Sea (age range 10-36 years) and 20 Iceland gill samples (age range 11-226 years) was quantified as 8-oxo-7,8-dihydroguanosine/ 10^6 guanosine and 8-oxo-7,8-2'-deoxyguanosine/ 10^6 deoxyguanosine respectively using a high-performance liquid chromatography coupled to electrochemical detection (HPLC-ECD) method [69]. This procedure is based on the high-salt nucleic acid release from proteins, followed by removal of proteins and fats by organic solvents at neutral pH, all in the presence of the metal chelator deferoxamine mesylate (DFOM) at 0°C. The samples were analyzed by HPLC coupled to electrochemical and UV detection (HPLC-ECD/UV). The chromatograms were recorded using the EZChrome Elite software (Scientific Software Inc., Pleasanton, CA, USA).

2.2.4 Measurement of protein stability

Protein stability was measured in 15 Baltic Sea (age range 11-35 years) and 25 Iceland animals (age range 11-226 years). According to the protocol developed by Pierce *et al.* [131] protein stability was measured to determine resistance to protein unfolding in cytosolic protein extracts from gill tissue. Proteins were incubated with urea followed by UV-induced crosslinking with 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt (BisANS). The labelled proteins were related to total protein content to yield the unfolded protein to total protein ratio. Gill tissue (150 – 200 mg) was homogenized in 350 μ l 1x labelling buffer (50 mM Tris-HCL, 10 mM MgSO₄, pH 7.4) mixed with protease inhibitor cocktail (Roche, Mannheim, Germany) and Zirconium Silicat Beads using a Retsch MM 301 homogenizer (2 min with 30/s). The lysates were ultracentrifuged in a Beckmann TL-100 ultracentrifuge for 1 hour at 100,000 x *g* at 4°C. Cytosolic protein concentration was determined using Pierce BCA assay (Thermo Scientific, Rockford, USA). The cytosolic extracts were diluted to 1 mg/ml in a 96 well plate and incubated with 1 M urea on ice for 1 hour. Afterwards 100 μ M BisANS was added and samples were incubated for 1 hour on ice under direct exposure of a 115-V 0.16-A handheld longwave UV lamp (365 nm, UVL-56, UVP, Cambridge, UK). Following photoincorporation, 1x reducing loading buffer was added to each sample and proteins were separated on SDS gel at 150 V. After electrophoresis, the gels were illuminated with 365 nm UV light and BisANS fluorescence was captured using the Molecular Imager ChemiDoc XRS Imaging System (BioRad, Munich, Germany) using the QuantityOne-4.6.8 software by BioRad. For the total protein content, gels were prepared as described above for soluble and insoluble protein carbonyls. Image analyses were also carried out in the same manner and related to total protein content.

2.2.5 DNA extraction

Frozen tissue was ground into a fine powder with mortar and pestle under liquid nitrogen atmosphere to prevent DNA and RNA degradation by DNases and RNases, respectively. Approximately 50 mg tissue powder was used to extract DNA using the DNeasy Blood&Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. This method is based on DNA isolation and purification over a silica membrane column in the presence of high concentrations of chaotropic salt. The elution volume was 100 μ l and incubated on the column for 5 min prior to elution.

2.2.5.1 Quality assessment

The DNA and RNA yield and quality were assessed using a NanoDrop ND-1000 Spectrophotometer (PEQLAB Biotechnology GmbH, Erlangen, Germany). The NanoDrop measures absorbances at 260 nm (for nucleic acid absorbance), 280 nm (for protein absorbance), and 230 nm (absorbance of disturbing contaminants, e.g. EDTA, carbohydrates, phenol). 260/280 ratios around 1.8 for DNA and 2.0 for RNA are generally referred to as "pure". Acceptable 260/230 ratios are commonly in the range of 1.8-2.2. The eluted DNA was stored at -20°C until further usage.

2.2.6 Measurement of telomere length

The telomere lengths of genomic DNA of gill tissue from 24 Baltic Sea animals (10-36 years) and 38 Icelandic animals (6-226 years) were measured using the *TeloTAGGG* Telomere Length Assay (Roche, Mannheim, Germany) according to the manufacturer's instructions. In this assay, 1 μ g of extracted DNA was digested by restriction enzymes (*HinfI* and *RsaI*) overnight in a 37°C heat block (Thermomixer compact, Eppendorf, Hamburg, Germany). The restriction enzymes provided in the assay kit digest all genomic sequences except for the 5'-TTAGGG-3' telomeric repeat sequence. Digested genomic DNA was separated from intact telomeres on a 0.8 % LE agarose gel run at 30 V for 5 hours (2.3.2.3). The separated DNA fragments were used for Southern blotting [157] (setup see Fig 2.4) where the DNA was transferred onto a nylon Amersham Hybond-N+ membrane (GE Healthcare, Buckinghamshire, UK) over-night and the DNA was fixed by baking the membrane in an oven (Memmert GmbH + Co. KG, Schwabach, Germany) at 80°C for 2 hours.

After hybridization and washing as described in the manual, the Southern blots were exposed to chemiluminescence for 20 min and visualized in a Molecular Imager

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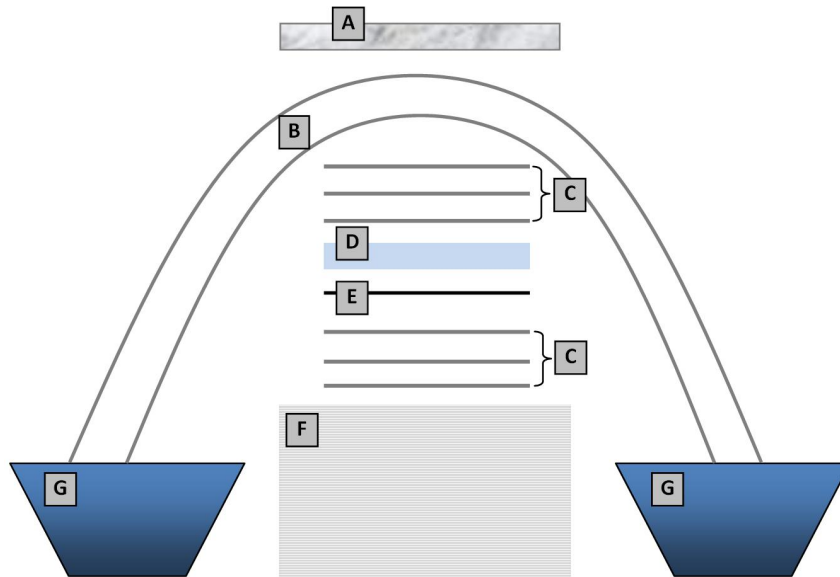


Figure 2.4: Setup for upside-down Southern blot: glass plate with weight on top (A), two long stripes of Whatman paper (B), three layers of Whatman paper (C), agarose gel with DNA (D), nylon membrane (E), stack of paper towel (F), pans with 20x SSC buffer (G). The layers of Whatman paper and nylon membrane were of similar dimensions as the agarose gel.

ChemiDoc XRS Imaging System (BioRad, Munich, Germany) with the BioRad QuantityOne-4.6.8 software. Fig 2.5 shows a Southern blot with telomeres detected as smears of undigested DNA resolved on the gel and the area of highest density that is determined as the length of the telomeres.

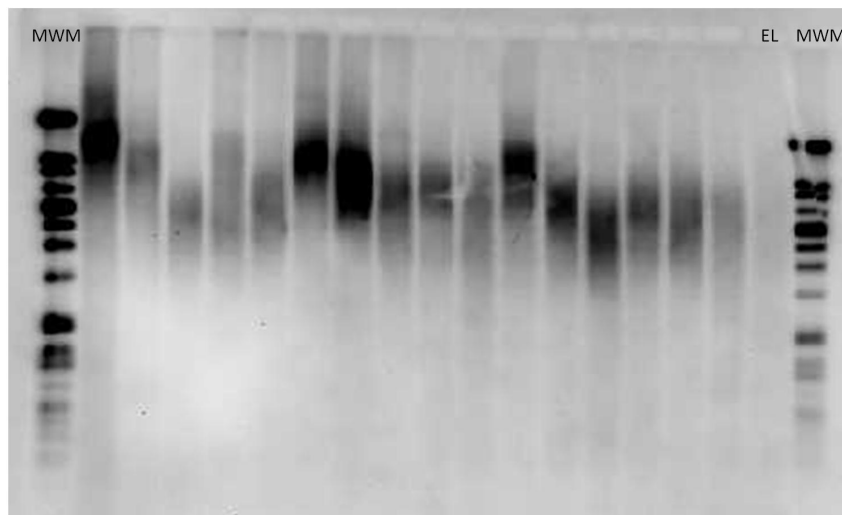


Figure 2.5: Southern blot for the detection of telomere lengths shown as smears of undigested genomic DNA. MWM = molecular weight marker, EL = empty lane

2.2.7 Measurement of telomerase activity

The telomerase activity was measured using the Quantitative Telomerase Detection Kit (US Biomax Inc., Rockville, MD, USA) from the gill tissues of 10 Baltic Sea and 15 Icelandic animals that were also used for telomere length determination. The telomerase activity was further measured in a tissue panel consisting of mantle, digestive gland, gill, and foot tissues from 3 of the same individuals per population. As per the manufacturer’s instructions, proteins were extracted from 40 mg of ground tissue lysed with 100-200 μ l lysis buffer provided with complete protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was determined using the DC Protein Assay (BioRad, Munich, Germany) according to the manufacturer’s instructions, with the exception that absorption was measured at 612 nm wavelength. Protein extracts were shock frozen in liquid nitrogen and stored in aliquots at -80°C until further analysis. The assay measures the telomerase activity from the extracted protein based on its ability to add telomeric repeats on a substrate oligonucleotide. The elongated substrate is then amplified and quantified in a qRT-PCR (Tab 2.1). The initial concentration of telomerase can be inferred from the number of amplified qRT-PCR products using a standard curve generated with a provided standard (TSR template).

Table 2.1: Reaction setup for telomerase activity assay.

Content:	Volume per reaction:
2x QTD Premix	6.25 μ l
Protein extract (175 ng)	1 μ l
dH ₂ O	5.25 μ l
<u>Temperature:</u>	<u>Time:</u>
25 $^{\circ}$ C	20 min
95 $^{\circ}$ C	10 min
95 $^{\circ}$ C *	30 s *
60 $^{\circ}$ C *	30 s *
72 $^{\circ}$ C *	30 s *

* x40 cycles

The incubation step prior to qRT-PCR at 25 $^{\circ}$ C for 20 min allows telomerase mediated extension of provided forward primers with telomeric repeat ends (substrate). Anchored reverse primers with modified telomere repeat sequences define the end of the telomerase product. The PCR amplification of the elongated product is initiated by a telomerase heat-inactivation step which simultaneously serves as activation for the hot start Taq polymerase. All samples were run in duplicates. A dilution series ranging from 0.04375-2.1 $\mu\text{g}/\mu\text{l}$ of protein extract including HeLa cell-extracts for positive controls and heat inactivated samples were pretested in the qRT-PCR assay. The provided TSR control templates were used to generate a standard curve

and find an optimal protein concentration of 175 ng/ μ l to detect telomerase activity. The TSR standard curve used for sample measurements had the following template concentrations:

Table 2.2: Standard concentrations of TSR control sample used for sample measurement.

Concentrations (amole/ μ l)	Number of molecules
1E-1	60000
2E-2	12000
8E-4	480
3.2E-5	20
6.4E-6	4

After generating a linear regression curve, the telomerase activity could be deduced from concentrations and number of molecules measured during qRT-PCR.

2.3 Differential gene expression analysis in both *A. islandica* populations

The immense difference of MLSP between the two populations of *A. islandica* was the cornerstone idea for an RNA sequencing experiment that should determine the differential gene expression of Baltic Sea animals compared to Iceland animals in general and along different ages.

2.3.1 Transcriptome strategy

To date there has been only one individual animal found in Iceland exceeding 400 years of age [181]. The MLSP for the Icelandic population was thus considered to be 300 years and defined as 100 % when calculating the relative lifespan of sampled Icelandic individuals. Among the Baltic Sea population, animals reaching the MLSP of 40 years [19] are more frequent, allowing us to consider this age as the 100 % relative lifespan for this population. Based on the MLSP and the oldest animal found in Iceland – reaching an age of 226 years – the ages of the different groups to be investigated for both populations were then calculated (Fig 2.6). *A. islandica* can be mature as young as 6 years old [170] but are generally fully mature at 10-12 years of age [169]. Therefore, to exclude the unnecessary analysis of expressed interfering maturation genes, the young aged reference group should therefore be at least 12

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years old, which corresponds to 30 % of the MLSP of the Baltic Sea population. The calculated 30 % MLSP for Icelandic animals approx. corresponds to the age of 100 years, which was used to define the middle aged group of the Iceland population (Fig 2.6). Additionally, to understand early differences between the two populations, young aged 12 year old Icelandic animals were also chosen to be sequenced. This resulted in 3 age groups (young, middle, and old) for the Icelandic and 2 age groups (young, old) for the Baltic Sea population.

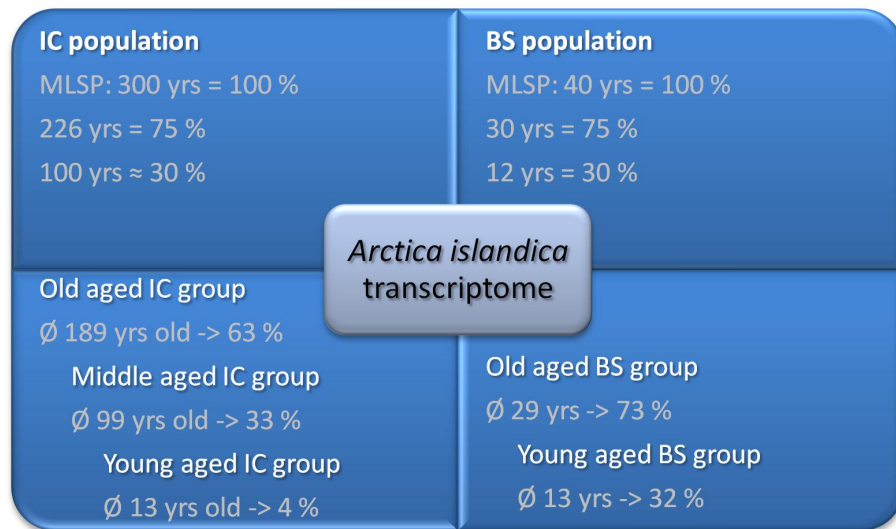


Figure 2.6: Age groups for transcriptome strategy (top two boxes) and samples for transcriptome generation (bottom two boxes) (IC=Iceland, BS=Baltic Sea, MLSP=maximum lifespan, yrs=years, Ø=average).

For a higher transparency, low, as well as high proliferating tissues were chosen (heart and gill respectively) [159] and sequences of each single animal were tagged (2.3.1.3) in order to retrace the origin of the single sequences. Since the majority of old Icelandic individuals were female and to prevent sex-specific variances in the analysis, all individuals chosen for RNA sequencing were female. Four female individuals for both tissues from each age group were chosen which yields a total of 40 individual transcriptomes sequenced.

2.3.1.1 RNA extraction

Frozen gill and heart tissues were ground with mortar and pestle under liquid nitrogen atmosphere to prevent RNA degradation by RNases. For RNA extraction β -mercaptoethanol was used with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA molecules >200 bp can be isolated by the provided RNeasy Mini Spin columns. An incorporated in-column DNase step

(QIAGEN RNase-Free DNase) was performed twice to ensure complete digestion of remaining DNA.

RNA from heart tissue was extracted using the TRI reagent: 600 μ l TRI-reagent with approx. 50 mg of ground tissue was vortexed to lyse the tissue, transferred to a QIAshredder Mini Spin Column and centrifuged at $>16,000 \times g$ for 2 min. The flow-through was transferred to a new vial and 120 μ l chloroform was added, vortexed, and incubated at room temperature for 5 min. The mixture was centrifuged at $>16,000 \times g$ at 4°C for 15 min and the upper aqueous phase was transferred into a new vial. Next, 300 μ l of a solution composed of 150 μ l Isopropanol, 75 μ l 0.8 M Sodium citrate, 75 μ l 1.2 M NaCl was added, mixed, incubated at room temperature for 5 min, and centrifuged at $>16,000 \times g$ at 4°C for 10 min. The pellet was washed with 500 μ l of 75 % EtOH, centrifuged at $>16,000 \times g$ for 1 min. After discarding the supernatant, the sample was centrifuged again and the remaining supernatant was carefully removed with a pipet. The pellet was dried and resuspended in 100 μ l RNase-free H_2O . After resuspension, 350 μ l RLT-Buffer was added and mixed before 250 μ l of 96 % EtOH was added. The final sample mix was transferred into RNeasy spin columns and the extraction proceeded as described in the RNeasy Mini Kit. The eluted RNA was stored at -80°C until further use.

2.3.1.1.1 Quality check with Bioanalyzer

The quality of the RNA extracted for sequencing (2.3.1.1) was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). In the fluorescent measure of total RNA, distinctly separated 18S and 28S RNA peaks indicate intact RNA whereas starting transitions of one peak to the other show degraded RNA. The stage of degradation is expressed as an RNA-integrity number (RIN). The procedure was performed as described by manufacturer's instructions.

2.3.1.2 Generation of ds cDNA with SMARTer Kit

For the Next-Gen sequencing ds cDNA was generated using the SMARTer PCR cDNA Synthesis Kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to manufacturer's instructions. The 3' SMART CDS Primer IIA anneals to the 3'poly-A-tail of the mRNA (coding sequence) thereby selecting for mRNA and depleting the dominating rRNA. A modified reverse transcriptase elongates the primer and attaches several cytidine residues to the product end only when reaching the 5'end of the template. The SMARTer II Oligonucleotide primer consists of a set of guanosines complementary to the attached cytidines and a known sequence to flank the elongated

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product. After annealing of this primer to the cytidine residues a so-called template switch of the reverse transcriptase occurs to complete the First-strand synthesis (Tab 2.3). The template switch is the addition of the known flanking sequence to the template (SMART= *Switching Mechanism At 5' End of RNA Transcript*).

Table 2.3: First-strand synthesis setup for SMARTer PCR.

Content 1:	Volume per reaction:
total RNA	x μl (1 μg)
3'SMART CDS Primer IIA (12 μM)	1 μl
RNase free dH ₂ O	add 4.5 μl
<u>Temperature:</u>	<u>Time:</u>
72°C	3 min
42°C	2 min
<u>Add to Content 1:</u>	<u>Volume per reaction:</u>
5x First-strand Buffer	2 μl
DTT (100 mM)	0.25 μl
dNTPs (10 mM)	1 μl
SMARTer II Oligonucleotide (12 μM)	1 μl
RNase Inhibitor	0.25 μl
SMARTScribe Reverse Transcriptase (100 U)	1 μl
<u>Temperature:</u>	<u>Time:</u>
42°C	90 min
70°C	10 min

The First-strand is now flanked by known sequences which serve as priming sites for the generation and amplification of the second strand during an end-to-end cDNA amplification step as outlined in Tab 2.4.

Table 2.4: PCR setup and temperature program for second strand synthesis and amplification of first strand cDNA products.

Content:	Volume per reaction:
First-strand synthesis product	2 μl
dH ₂ O	82 μl
10x Advantage 2 PCR buffer	10 μl
50x dNTP Mix (10 mM of each dNTP)	2 μl
5'PCR Primer IIA (12 μM)	2 μl
50x Advantage 2 Polymerase Mix	2 μl
<u>Temperature:</u>	<u>Time:</u>
95°C	1 min
95°C *	15 s *
65°C *	30 s *
68°C *	6 min *
4°C	hold

* x13 cycles

The ds cDNA products were purified using the QIAquick PCR Purification Kit

(Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and quality of the ds cDNA were assessed with the NanoDrop ND-1000 Spectrophotometer (2.2.5.1) and by agarose gel electrophoresis (2.3.2.3).

2.3.1.3 FLX runs

A cDNA library was prepared with the cDNA Rapid Library Preparation Kit (Roche, Mannheim, Germany) for the GS FLX Titanium Series according to manufacturer's instructions. With the addition of biotinylated adaptor sequences the previously fragmented cDNA is captured onto streptavidin coated magnetic beads under conditions that favor one fragment per bead. Incorporated into the adaptor sequences is a so-called barcode sequence so that all created sequences of the 40 transcriptomes (see 2.3.1) could be traced back to its origin. The DNA library was amplified using the emPCR-Lib-L SV Kit (Roche, Mannheim, Germany) for GS FLX Titanium Series according to manufacturer's instructions. The pyrosequencing method, which relies on the detection of pyrophosphate by nucleotide incorporation, was performed with the GS FLX Titanium Chemistry (454 Life Sciences, Branford, CT, USA) according to the Sequencing Method Manual for the GS FLX Titanium Series.

2.3.1.4 Bioinformatic assembly and annotation

The resulting FLX sequences were extracted from FLX output files with the `sffinfo` script (Roche, Mannheim, Germany). The sequences were quality controlled by trimming SMART primers, sequencing adapters, poly-A-tails, and low quality sequence ends. Furthermore, only reads ≥ 40 bp were used for the assembly process. As a first step the Celera Assembler [111] was used for the Baltic Sea assembly and the GS De Novo Assembler Software NEWBLER by Roche for the Iceland assembly. Sequences from all 40 single transcriptomes were assembled into one complete combined transcriptome database. However, to accelerate the assembly process and reduce sequence complexity, sequences from the two populations were assembled separately and referred to as population specific transcriptomes (referred to as BS_transcriptome for Baltic Sea specific and IC_transcriptome for Iceland specific in this work). All subsequent assembly steps were performed using the TG-ICL assembler (CAP3) in several rounds. The resulting contigs from the population specific transcriptomes were further mapped onto an already existing transcriptome [130] in a second step to generate the combined final RNA seq database (referred to as combined_transcriptome in this work) (Fig 2.7). In a final step all available reads were mapped onto the final transcriptome to obtain gene expression data.

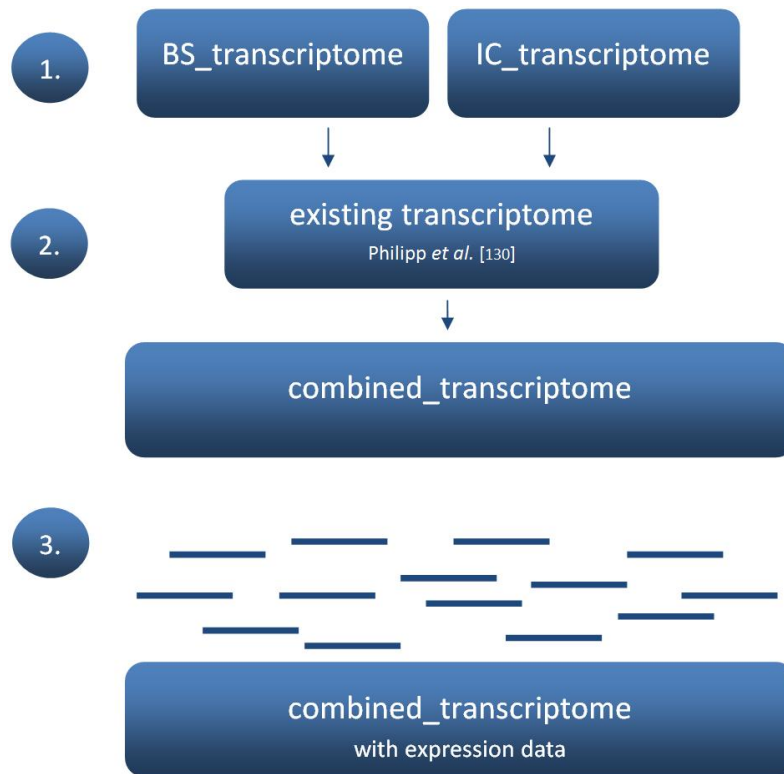


Figure 2.7: Assembly steps for the final combined_transcriptome showing population specific transcriptomes in step 1, mapping onto the already published transcriptome [130] in step 2 and further mapping of all reads onto the combined_transcriptome in step 3 to obtain expression data (BS=Baltic Sea, IC=Iceland).

The combined final RNA database contains contigs assembled from all single RNA sequenced 40 transcriptomes plus the contigs of the published transcriptome [130]. Annotation of putative genes, proteins and domains of the assembled contigs was done using the blastx algorithm against UniprotKB/Swissprot. Conserved domains were identified via InterProScan [197] and Gene Ontology (GO) terms were deduced from blast and InterProScan results. All information (contigs, associated information, contig coverage, etc.) was uploaded into the transcriptome analysis and comparison browser, T-ACE [127], for further analysis.

2.3.1.5 Gene search via keywords

The incorporated keyword-search-function in the T-ACE browser was used to look up a number of relevant putative genes: genes important for the immune system, stress response, apoptosis, and autophagy, as well as known ageing-relevant genes and genes involved in DNA repair and the antioxidant system. Several factors such as the E-value, and more importantly gene-specific domains observed in one contig, were considered

for the true identification of genes. To achieve this, contig nucleotide sequences were blasted against UniProtKB/Swiss-Prot (<http://www.uniprot.org/help/uniprotkb>) and the NCBI nr/nt database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Protein open reading frames were blasted against SMART (<http://smart.embl-heidelberg.de/>) [148] and NCBI DART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>) [56] to obtain protein domains. Gene-specific or relevant protein domains were matched with domains found in homologous genes at NCBI HomoloGene (<http://www.ncbi.nlm.nih.gov/homologene>). The sequence verification of identified genes was performed as outlined in 2.3.2.5.

2.3.1.6 GO-term and KEGG-pathway analysis with T-ACE

The genes and associated functions that are rather enriched or depleted in the groups of each population are determined from the generated sequences using the “run-compare” tool of the T-ACE program [127]. These analyses were done for KEGG-pathways and GO-terms which provide the higher or lower abundance of the different pathways and GO-categories (biological process, cellular component, molecular function) in the groups that are compared. For instance, contigs that were only covered by reads from Baltic Sea individuals (Baltic Sea specific) were compared to all Baltic Sea contigs which were covered by reads from Baltic Sea and also Iceland individuals. This gives information on whether contigs that are solely expressed by Baltic Sea animals show a higher functional abundance or enrichment in certain pathways. The same was done for the Iceland population as well as comparing gill and heart tissues and the different age groups.

2.3.1.7 Expression profile analysis

For some genes the expression greatly varies over age. For instance, genes relevant in embryonic development are strongly expressed during early stages of development but are completely silenced during adulthood of an organism. Figure 2.8 below depicts the possible gene expression profiles over age. These expression profiles were assessed for the three different age groups collected in Iceland and the two in the Baltic Sea. Four biological replicates (individual animals) were used to represent each group in the transcriptome (section 2.3.1). Since significant differences in gene expression between two age groups could be due to the low number of replicates ($n=4$) not identified, gene expression analysis was, thereby, based on distinctly different expressed contigs and conducted as described below.

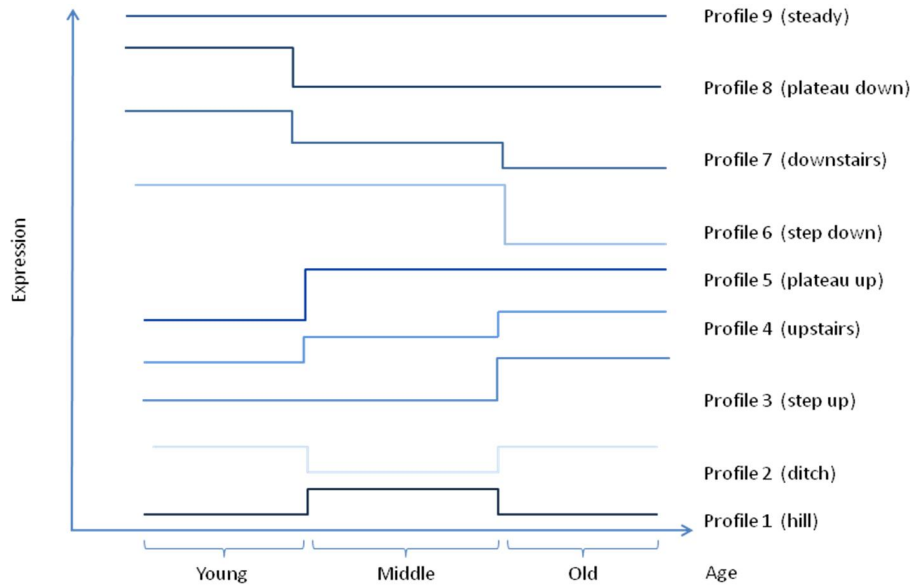


Figure 2.8: Possible expression profiles of a gene over age.

2.3.1.7.1 Used statistics to analyze expression profiles

Normalization within each sample was performed by calculating the RPKM-value (reads per kilobase per million mapped reads) [108], which normalizes the contigs according to the number of reads and transcript length. This was done for all tagged individuals according to equation (2.1).

$$\text{RPKM} = \frac{\text{no. of reads}}{\text{length of contig}} * 1000 \quad (2.1)$$

A normalization between samples was performed using “normalizeBetweenArrays” function in the limma-package of R [46]. In this calculation the expression values are normalized to achieve consistency between arrays. The method=“quantile” was chosen for this procedure. The normalized database was uploaded into T-ACE and the “Database statistics”-tool [127] was used to compare age groups of one population within one tissue or in both tissues combined. The following parameters were selected:

Expression analysis / Fold change / Choose all contigs / Min contig length:100 / Min number of reads:20 / Min fold change:1 / median, absolute / Max pValue:0.05 / distinct:100%

The resulting table was exported into Excel where a correction of p-values, using multiple testing [21] was performed before distinct differential expression between the analyzed groups was investigated.

2.3.2 Detailed expression analysis with qRT-PCR

The detection of PCR products during their production in real time, via incorporation of the DNA intercalating fluorescent dye SYBR green, and the subsequent deduction of the initial amount of template briefly describes the concept of quantitative real-time PCR (qRT-PCR). With the formation of new double-stranded products the fluorescent emittance of SYBR green increases each cycle and is measured in real time. The number of cycles needed to reach a certain threshold (cycle threshold = CT-value) is used to calculate the initial amount of template. In expression analysis, gene expression is relatively quantified using one or several housekeeper genes, which are ubiquitously expressed, as a reference.

Genes from different categories – seven genes involved in DNA repair, four ageing-relevant genes, three genes coding for antioxidants and four housekeeper genes (GAPDH, β -Actin, 18S, 28S) – were selected for detailed expression analysis (Tab 3.2, see Appendix e1 Primer Sequences for sequences). The expression of the total 18 genes was investigated in gill tissue of 20 Baltic Sea and 25 Iceland animals using the 7900HT Fast Real-Time PCR System as described in the following.

2.3.2.1 Primer design

The amplification of a selected nucleotide sequence via polymerase-chain-reaction (PCR) using the enzyme DNA polymerase is one of the fundamental techniques in molecular biology. As DNA polymerase requires a double-stranded (ds) nucleotide substrate as starting point, short oligonucleotides, priming the sequence of interest (primers), are designed specifically annealing only to the desired sequence. For the selected genes (3.3.1, Tab 3.2), primers were designed using the available online (<http://frodo.wi.mit.edu/primer3/>) open source software Primer3 [141]. In order to amplify the transcripts and verify their assembled sequence in the transcriptome by Sanger sequencing 2.3.2.5, full length primers that cover at least 83 % of the annotated contigs were designed for each gene. To investigate the detailed gene expression via qRT-PCR, another set of primers was designed to generally cover 100-400 bp of the gene. All primers were checked for low ΔG values to prevent formation of local self-complementarity (hairpins) and the formation of homodimers and heterodimers using OligoAnalyzer 3.1 (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>) with standard settings. The primers were also blasted in the T-ACE final combined_transcriptome to ensure specific binding to the requested transcript only. The qRT-primers were designed with an annealing temperature of 60°C.

2.3.2.2 Primer testing

Each primer was tested in an initial gradient PCR using the GoTaq polymerase (Promega, Mannheim, Germany) or Advantage Taq polymerase (BD, Heidelberg, Germany). The reaction setup and temperature programs of the gradient PCR is stated in Tab 2.5.

Table 2.5: PCR reaction setup and temperature program for (A) GoTaq polymerase and (B) Advantage Taq polymerase.

(A)		(B)	
Content:	Volume per reaction:	Content:	Volume per reaction:
Template	1 μ l	Template	1 μ l
5x buffer	4 μ l	10x buffer	2 μ l
dNTPs (10mM)	0.5 μ l	dNTPs (10mM)	0.5 μ l
Primer (10 μ M) each	0.25 μ l	Primer (10 μ M) each	0.5 μ l
GoTaq (5u/ μ l)	0.2 μ l	Advantage Taq (2.5 u/ μ l)	0.25 μ l
dH ₂ O	13.8 μ l	dH ₂ O	15.75 μ l
<u>Temperature:</u>	<u>Time:</u>	<u>Temperature:</u>	<u>Time:</u>
95°C	5 min	95°C	5 min
95°C *	30 s *	95°C *	30 s *
annealing temp. gradient *	30 s *	annealing temp. gradient *	30 s *
72°C *	60 s per 1 kb *	68°C *	60 s per 1 kb *
72°C	7 min	68°C	7 min
4°C	hold	4°C	hold

* x40 cycles

PCR product sizes were verified on a 1 % agarose gel (2.3.2.3) against the SmartLadder MW-1700-10 and purified when required (see 2.3.2.4)

2.3.2.3 Agarose gel electrophoresis

DNA (and RNA in some cases) and PCR product sizes were checked by agarose gel electrophoresis (1 % LE agarose). This method separates nucleotide molecules according to their size in an electric field. The agarose gel was run in 0.5x TAE running buffer at constant voltage using a BioRad Power Pac 300 (BioRad, Munich, Germany) power supply. The SmartLadder MW-1700-10 (Eurogentec, Seraing, Belgium) was used as a size marker. The DNA-intercalating agent SYBR Safe DNA gel stain (Invitrogen, Karlsruhe, Germany) was used to visualize DNA samples upon exposure to UV light in the Molecular Imager ChemiDoc XRS Imaging System (BioRad, Munich, Germany).

2.3.2.4 Purification of PCR products

For further sequence analysis, resolved PCR products were cut from the agarose gel with a scalpel, transferred into reaction tubes and extracted from the gel using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Mannheim, Germany) according to manufacturer's instructions. This procedure is a silica membrane column based purification method. In some cases, PCR products were enzymatically purified prior to sequencing using to the reaction setup in Tab 2.6.

Table 2.6: Reaction setup for the enzymatic PCR product purification.

Content:	Volume per reaction:
PCR Mix	5 μ l
Exonuclease I (20 u/ μ l)	0.5 μ l
Shrimp Alkaline Phosphatase (1 u/ μ l)	1 μ l
<u>Temperature:</u>	<u>Time:</u>
37°C	15 min
85°C	15 min

In this enzymatic reaction, the Exonuclease I removes remaining primers by degrading single-stranded DNA in a 3'→5' direction and releasing deoxyribonucleoside 5'-monophosphates in a stepwise manner. Shrimp Alkaline Phosphatase catalyzes the release of 5'- and 3'-phosphate groups from DNA, RNA and nucleotides preventing further elongation or incorporation of dNTPs.

2.3.2.5 Sequence verification by Sanger sequencing

Sequences of assembled contigs selected for qRT-PCR were verified by Sanger sequencing [144]. This method relies on the chain-termination of DNA polymerase by incorporating specifically labeled ddNTPs. Sequences of up to 1,000 bp can be read by Sanger sequencing resulting in high quality sequences. In case of amplification of products for sequence verification, the Advantage Taq polymerase (setup see 2.3.2.2 Tab 2.5B) was chosen as it also contains a proofreading function – specifically, a 3'-5' exonuclease activity that allows excision of incorrect base pairs followed by insertion of correct base pairs. After clean up and purification of the PCR product (2.3.2.4) a sequencing PCR reaction with the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was performed as outlined in Tab 2.7.

The detection via capillary electrophoresis and conversion of the truncated products into sequences were performed with an ABI 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA). Obtained sequences were analyzed and evaluated

2 Materials and Methods

Table 2.7: Reaction setup and temperature program for sequencing PCR.

<u>Content:</u>	<u>Volume per reaction:</u>
PCR Product	2 μ l
5x Sequencing Buffer	1.85 μ l
Primer (3.2 μ M) each	1 μ l
dH ₂ O	4.8 μ l
Big Dye	0.35 μ l
<u>Temperature:</u>	<u>Time:</u>
96°C	1 min
96°C *	10 s *
50°C *	5 s *
60°C *	4 min *
4°C	hold

* x25 cycles

using Sequencher 5.0 to align contig, primers and generated sequences for sequence verification.

2.3.2.6 Generation of ss cDNA

For the expression analysis via qRT-PCR, ss cDNA was generated with the RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manual. In the following reaction setup, 1 μ g of extracted total RNA (2.3.1.1) was used:

Table 2.8: Reaction setup and temperature program for ss cDNA synthesis.

<u>Content 1:</u>	<u>Volume per reaction:</u>
total RNA	x μ l (1 μ g)
Oligo (dT) Primer	0.125 μ l
dNTPs (10 nM)	0.5 μ l
dH ₂ O	add 7.5 μ l
<u>Temperature:</u>	<u>Time:</u>
65°C	5 min
<u>Add to Content 1:</u>	<u>Volume per reaction:</u>
5x RT buffer	2 μ l
RevertAid Premium Enzyme Mix	0.5 μ l
<u>Temperature:</u>	<u>Time:</u>
25°C	10 min
50°C	15 min
85°C	5 min

The reaction volume was adjusted to yield a cDNA concentration of 100 ng/ μ l. The cDNA was stored at -20°C until further use.

2.3.2.7 qRT-primer testing

All qRT-primers were initially assessed in a PCR (2.3.2.2) and product purity and size were checked on an agarose gel (see 2.3.2.3). A preliminary qRT-PCR run was performed to check primer specificity by excluding the measurement of primer dimers and by-products not found by blasting primers against the combined_transcriptome database. These runs were performed in triplicates using a 2 ng/ μ l template pool of cDNA from Baltic Sea as well as Iceland animals. The qRT-PCR was performed according to Tab 2.9 in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The Real-Time PCR System was programmed and sequences were recovered using the Sequence Detection System 2.3 software provided by Applied Biosystems.

Table 2.9: qRT-PCR reaction setup and temperature program.

Content:	Volume per reaction:
template	5 μ l
SYBR Select Master Mix	4.5 μ l
Primer (10 μ M) each	0.25 μ l
<u>Temperature:</u>	<u>Time:</u>
50°C	2 min
95°C *	2 min *
95°C *	15 s *
60°C *	1 min *
4°C	hold

* x40 cycles

2.3.2.7.1 Dilution series for primer efficiencies

All qRT-primers were assessed for most efficient primer performance to ensure linear amplification of the target sequence. To achieve this, a dilution series of pooled cDNA was tested by qRT-PCR for each qRT-primer pair. Different template concentrations (ranging from 0.3125 to 25 ng per reaction) were tested in triplicates according to the qRT-PCR setup stated in Tab 2.9. The primer efficiencies (E) were calculated according to equation (2.2).

$$E = 10^{\frac{(-1)}{(\text{slope of the dilution curve})}} \quad (2.2)$$

The final qRT-primers and corresponding primer efficiencies are listed in Results Tab 3.6 and qRT-primers sequences are listed in Appendix e1 Primer Sequences.

2.3.2.8 Evaluation of qRT-PCR runs

The qRT-PCR runs were evaluated using the Δ CT-method for the relative quantification of gene expression. In this method, expression of the gene of interest is normalized to CT-values of a ubiquitously-expressed, stable housekeeper gene. All values were corrected by primer-specific efficiency values that had been assessed from dilution series described in 2.3.2.7.1 (efficiencies see 3.4.2, Tab 3.6). To find the most suitable housekeeper gene, qRT-PCR CT-values of the four housekeeper genes (GAPDH, β -Actin, 18S, 28S) were transformed into linear values with respective efficiencies and checked using NormFinder.xla, an Add-In for Microsoft Excel [5]. Normfinder indicated 28S as the most suitable housekeeper gene with best stability value.

2.4 Statistics

Data evaluation and further statistical analyses such as t-tests, calculation of linear models, Mann-Whitney test and determination of outliers using Grubb's test were performed using GraphPad Prism 5 Version 5.04. Data was checked for Gaussian distributions with normal t-test, and homogeneity of variances was checked with the F-test. If data were not normally distributed or variances were inhomogeneous, data were log or square-root transformed. If normal distribution could not be achieved, significances were calculated using the Mann-Whitney test. GraphPad Prism 5 designates statistical significances as followed: $p \leq 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). In column charts, data are expressed as means \pm SEM, unless otherwise indicated.

All used reagents and chemicals are listed separately in the back of this work in Appendix p1.

3 Results

3.1 Population characteristics

A. islandica mollusks live on the continental shelves across the North Atlantic Ocean where it survive in very different habitats. The animals' exposure to the diverse environments can be reflected in their physical appearance and morphology. At the moment it is not clear which environmental parameters are responsible for these differences. An investigation of the Baltic Sea and Iceland mollusk populations according to several physical parameters was a first step towards their characterization.

3.1.1 Growth curve

The populations' features were initially assessed by the measurement of morphometric parameters, sex- and age determination. Fig 3.1 shows the cross-section with indicated age-rings of the shell from the oldest *A. islandica* individual sampled from the Iceland population. The line of strongest growth (LSG), a commonly accepted size parameter, was used with the age of the animals to create the growth curve in Fig 3.2. Age determination for the Baltic Sea revealed an age range of 10-36 years, while from Iceland, the youngest animal was determined to be 6 years and the oldest 226 years of age. The Icelandic growth curve seems to reveal "recruitment gaps" with low numbers of offspring every 30-40 years. For both populations the growth curve (Fig 3.2) first rises exponentially and then starts to flatten. For the Icelandic animals the growth curve plateaus just before 40 years of age, whereas this is barely detected for the Baltic Sea population. The growth curve clearly reveals that the oldest animal of 226 years is not the largest, and that the deduction of age from size is inaccurate and unreliable. Other measured shell parameters (length, width, weight, see 2.1.2) did not correlate with age neither (data not shown), which argues for using annual ring counts for determining the exact individual age.



Figure 3.1: Cross section of the shell of the oldest found Iceland *A. islandica* individual (IC337) reaching an age of 226 years. Age rings are marked with years of lifespan.

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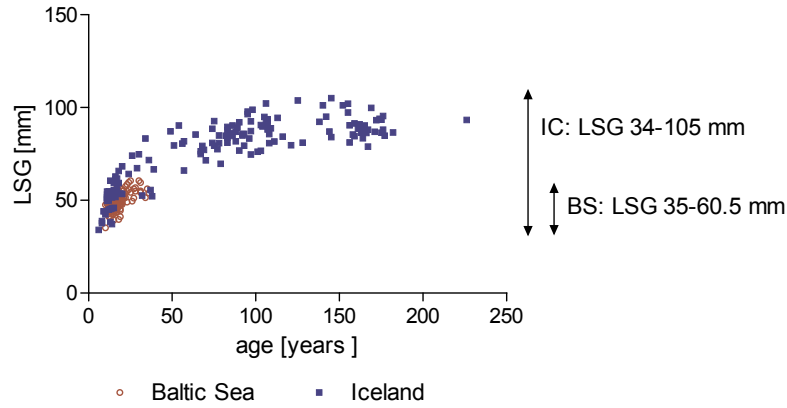


Figure 3.2: Growth curve of Baltic Sea and Iceland individuals showing the line of strongest growth (LSG) to indicate size and corresponding ages. Size ranges for each population (BS=Baltic Sea, IC=Iceland) are indicated at the right.

3.1.2 Sex distribution

Sexes could be determined for a total of 147 Baltic Sea animals of which 67 were female, and 80 were male. The Icelandic population was dominated by females (120 females, 72 % vs. 46 males, 28 %) whereas in the Baltic Sea population, the sexes were evenly distributed (female: 54 %, male: 46 %). Interestingly, the older Icelandic individuals were predominantly females, which confirms findings by Thorarinsdottir & Steingrímsson [170], while the Baltic Sea male and female groups had similar age distribution (Fig 3.3A).

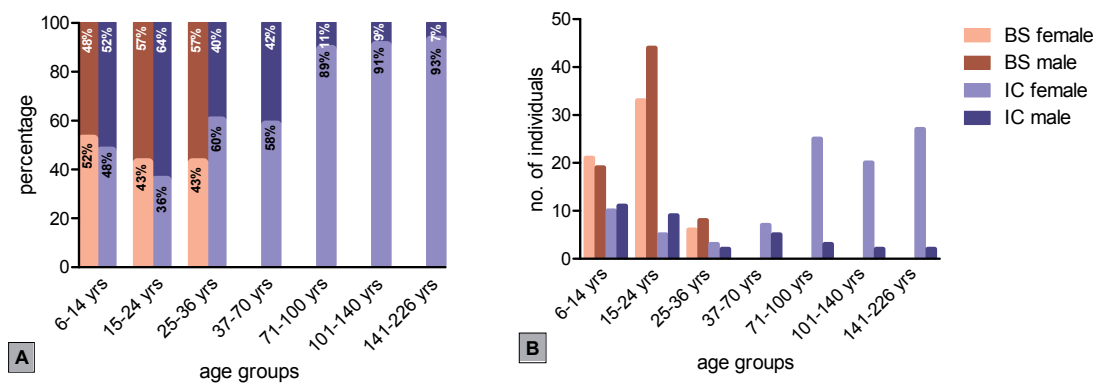


Figure 3.3: Sex distribution of the Baltic Sea (BS) and Iceland (IC) population in different age classes in percentage (A) and number of individuals (B).
 $n_{BS,female} = 67$; $n_{BS,male} = 80$; $n_{IC,female} = 120$; $n_{IC,male} = 46$

The female-to-male ratios were comparable between the two populations within the lifespan of the Baltic Sea population. However, since most of the collected Icelandic

animals reached an age beyond the Baltic Sea lifespan (Fig 3.3B) and the number of young individuals was scarce this may be a sampling artifact.

3.2 Analysis of ageing markers

There are a number of parameters considered as commonly accepted “ageing markers”, including oxidation of proteins, lipids, and nucleic acids. A selection of these biochemical parameters was investigated over age in the two studied *A. islandica* populations with the extreme differences in MLSPs (2.2).

3.2.1 Protein oxidation and stability

In the Iceland and Baltic Sea population, protein carbonyl content was quantified in gill tissues of sampled animals as a measure of oxidative damage to proteins. Fifteen Baltic Sea animals (age range 11-35 years) and twenty-five Icelandic animals (age range 11-226 years) were investigated. The results show that the oxidation level of soluble as well as insoluble proteins did not change over age in both populations (Fig 3.4). Calculated linear regression lines had a slope not significantly different from zero. Indeterminable results were not included in the analysis.

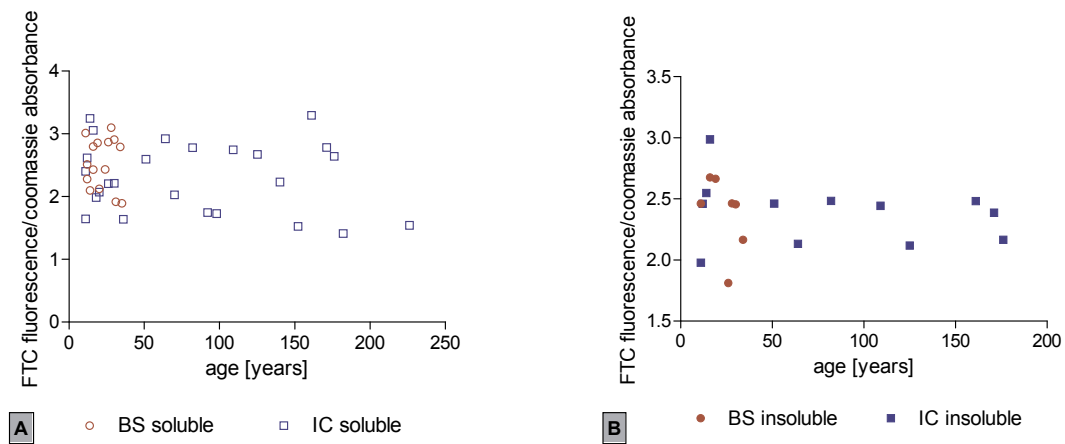


Figure 3.4: Relative quantification of protein carbonyls (in FTC fluorescence) to total protein content (coomassie absorbance) in the Baltic Sea (BS) and Iceland (IC) populations in soluble (A) and insoluble (B) proteins over age. $n_{BS,soluble} = 15$; $n_{IC,soluble} = 25$; $n_{BS,insoluble} = 7$; $n_{IC,insoluble} = 12$.

To measure protein stability the proteins extracted from gill tissues were stressed

3 Results

with urea and subsequently coupled with BisANS, a sensitive, non-polar probe for partially folded intermediates in protein-folding pathways. Fig 3.5 shows stable protein sensitivity over age to unfolding stress in both the Baltic Sea and Iceland populations.

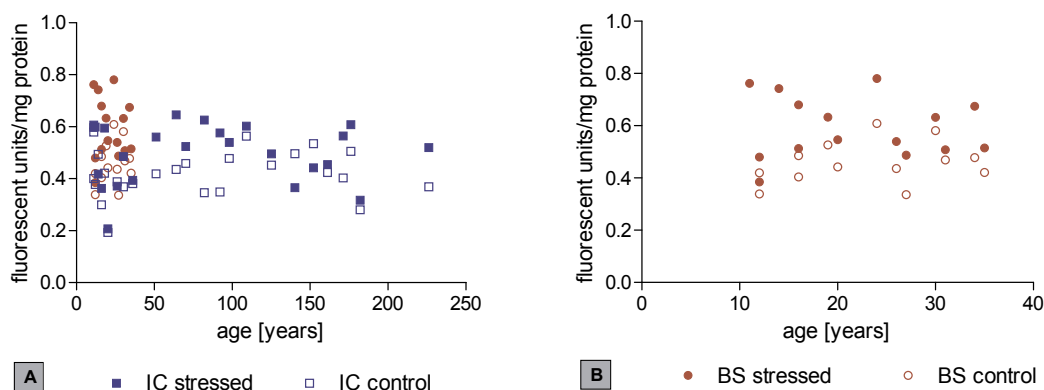


Figure 3.5: Protein stability over age in Iceland (IC) and Baltic Sea (BS) sampled populations. Protein stability was measured in fluorescent units from coupled BisANS probe relative to total protein content (A). The results for Baltic Sea samples are also shown separately (B). $n_{IC} = 25$; $n_{BS,stressed} = 15$; $n_{BS,control} = 13$.

A difference in levels of protein carbonyls between the two populations was not observed (mean values from 2.3-2.5 FTC fluorescence/coomassie absorbance) (Fig 3.6A). However, significant differences ($p < 0.01$) in protein stability were detected between stressed and non-stressed samples within each population (Fig 3.6B), but not between populations.

3.2.2 Lipid peroxidation

F₂-isoprostanes were quantified as markers for lipid peroxidation in collaboration with the laboratory of Steven Austad, Barshop Institute for Longevity & Aging Studies at the UT Health Science Center in San Antonio, USA. Fifteen Baltic Sea (age range 11-31 years) and twenty-five Icelandic animals (age range 11-226 years) were investigated. The results show that in the Baltic Sea as well as Iceland populations the lipid peroxidation over age stayed stable (Fig 3.7A). However, the mean levels of lipid peroxidation (F₂-isoprostanes) were significantly higher ($p < 0.0001$) in the Iceland population compared to those in the Baltic Sea population (Fig 3.7B).

3 Results

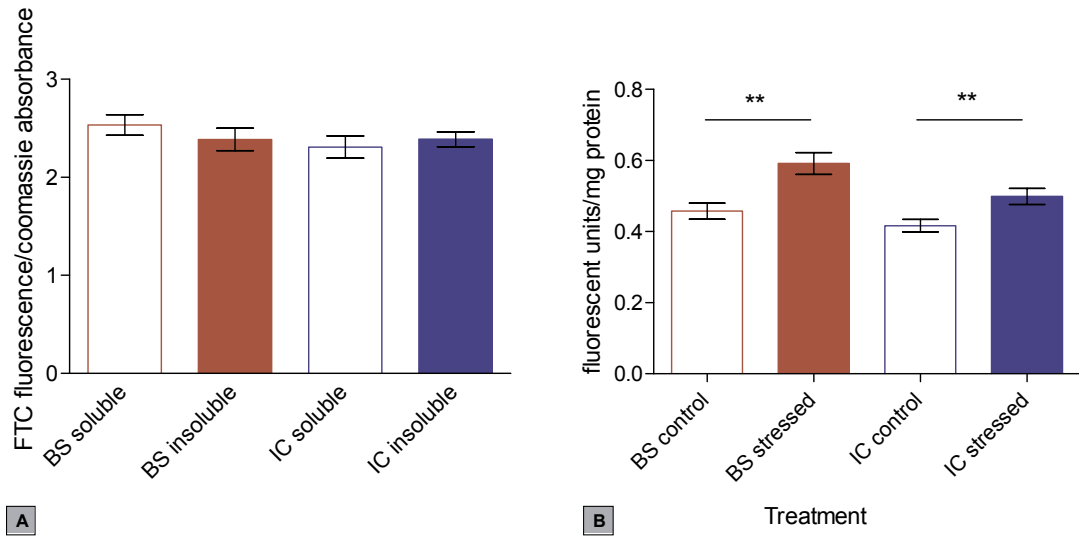


Figure 3.6: Levels of protein carbonyls (A) and protein stability (B) of Baltic Sea (BS) and Iceland (IC) samples. Mean values \pm SEM. $n_{BS,soluble} = 15$; $n_{BS,insoluble} = 7$; $n_{IC,soluble} = 25$; $n_{IC,insoluble} = 12$; $n_{BS,stressed} = 15$; $n_{BS,control} = 13$; $n_{IC} = 25$. ** indicate significance ($p < 0.01$).

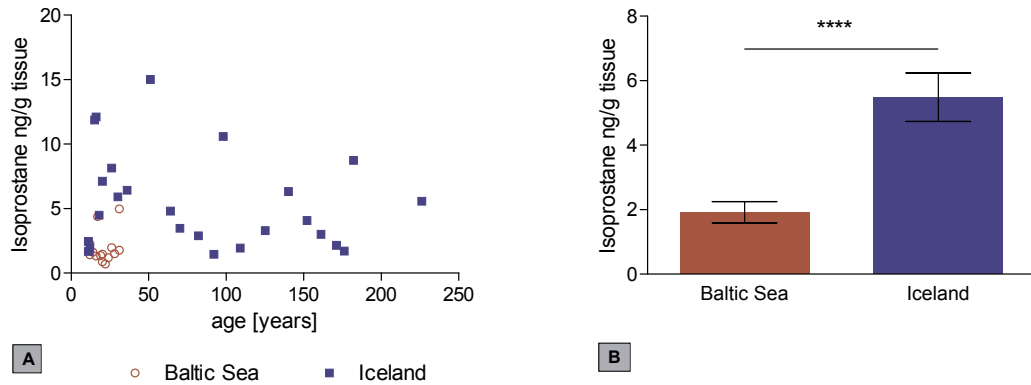


Figure 3.7: Lipid peroxidation (quantified as F₂-isoprostanes relative to total tissue weight) over age in Baltic Sea (BS) and Iceland (IC) populations (A). Mean levels of lipid peroxidation in the Baltic Sea and Iceland population (B). Mean values \pm SEM. $n_{BS} = 14$; $n_{IC} = 25$. **** indicates significance ($p < 0.0001$).

3.2.3 DNA and RNA oxidation

DNA and RNA oxidation in gill tissues of both populations was measured in collaboration with the laboratory of Stephanie Wohlgemuth, University of Florida. Samples from 21 Baltic Sea animals (age range 10-36 years; 19 females and 2 males) and 20 Iceland animals (age range 11-226 years; all females) were investigated (Fig 3.8).

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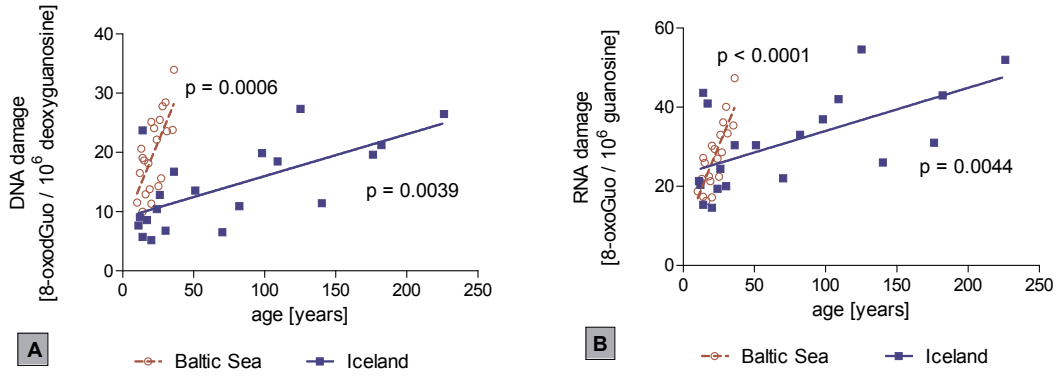


Figure 3.8: DNA (A) and RNA (B) oxidation in gill tissues of Baltic Sea and Iceland animals. Significance of the slopes (increase of damage over age) is indicated. $n_{BS} = 21$; $n_{IC} = 20$

DNA as well as RNA oxidation increased significantly over absolute lifespan in both sampled populations (Fig 3.8). However, both types of damage accumulated significantly faster in the Baltic Sea population than in Iceland, as indicated by the differences of the slope, $p_{DNA} = 0.0017$ and $p_{RNA} = 0.0014$, respectively. The corresponding linear models are:

$$Y_{DNA,BS} = 0.5827x + 7.226 \quad Y_{DNA,IC} = 0.07073x + 8.932$$

$$Y_{RNA,BS} = 0.8814x + 8.168 \quad Y_{RNA,IC} = 0.1089x + 23.1$$

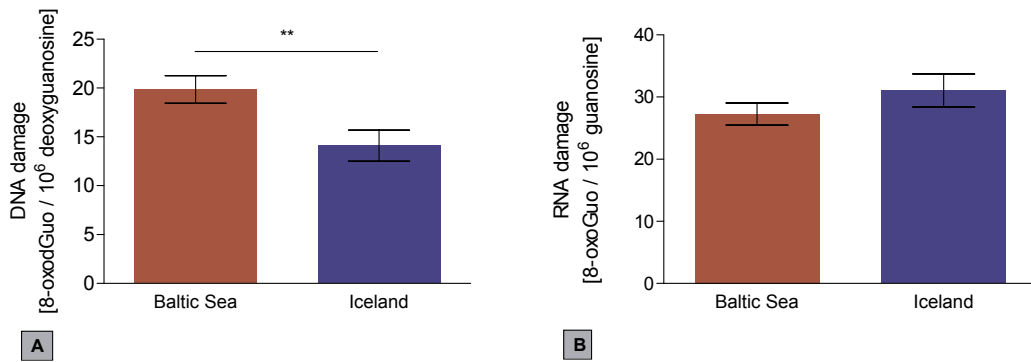


Figure 3.9: Mean levels of DNA (A) and RNA (B) oxidation in sampled Baltic Sea and Iceland animals. Mean values \pm SEM. $n_{BS} = 21$; $n_{IC} = 20$. ** indicates significance by t-test ($p=0.0099$).

The mean levels of RNA oxidation between the two sampled populations were comparable (Fig. 3.9B), but DNA damage level was significantly (t-test, $p=0.0099$) higher in the Baltic Sea animals compared to Icelandic animals (Fig 3.9A). Remarkably, regarding the relative lifespan of the investigated populations, damage accumulated at the same pace in both populations (Fig 3.10).

3 Results

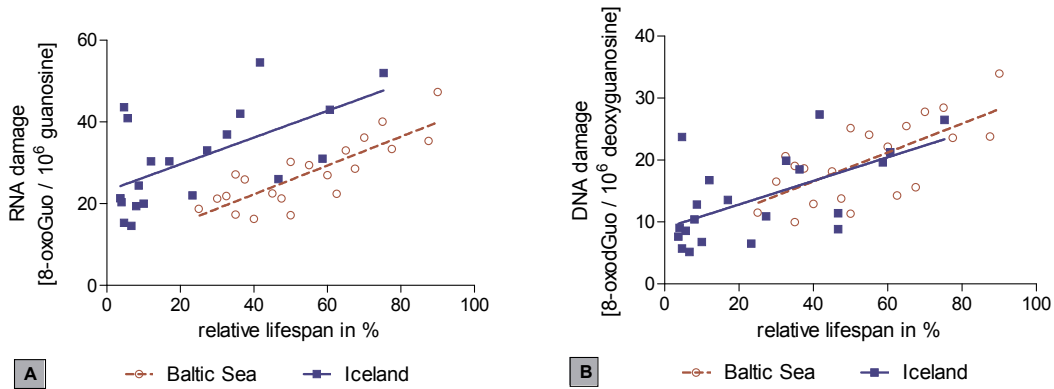


Figure 3.10: RNA (A) and DNA (B) oxidation increase with a comparable slope over relative lifespans of Baltic Sea and Iceland population. $n_{BS} = 21$; $n_{IC, RNA} = 20$; $n_{IC, DNA} = 21$.

3.2.4 Telomere length

Telomere length was measured in gill tissue of 24 Baltic Sea (15 females, 7 males, 2 undetermined sex) and 38 Icelandic (32 females, 5 males, 1 undetermined sex) animals across the complete investigated lifespan of the Baltic Sea (10-36 years) and Iceland (6-226 years) populations (Fig 3.11).

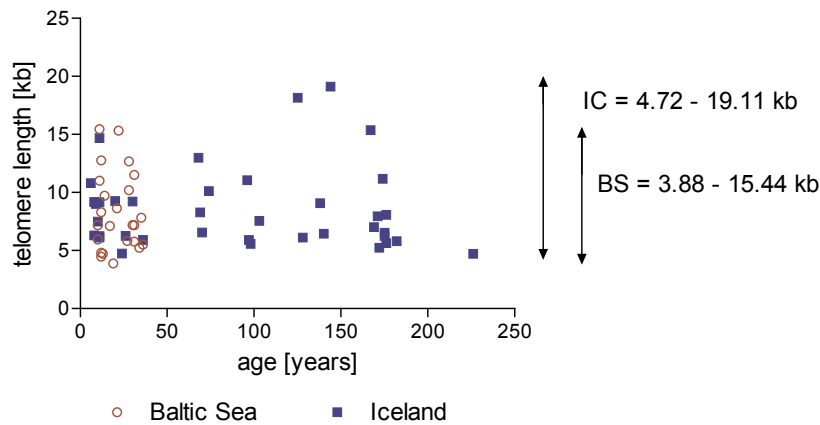


Figure 3.11: Telomere length in gill tissue of Baltic Sea (BS) and Iceland (IC) individuals over the investigated life span. $n_{BS} = 24$; $n_{IC} = 38$.

In the sampled *A. islandica* animals, the telomere length strongly varies independently from age or population indicating high heterogeneity. In young and old animals, telomeres ranged from 4.72 to 19.11 kb for the Icelandic population, and from 3.88 to 15.44 kb for the Baltic Sea population. Therefore, no significant differences in telomere lengths between Iceland and Baltic Sea populations were observed ($\text{mean}_{IC} = 8.55$ kb vs. $\text{mean}_{BS} = 8.26$ kb; $p = 0.65$). Additionally, in both

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populations telomere length did not correlate with age (linear model $y_{IC} = -0.0013x + 8.6701$; $y_{BS} = -0.0344x + 8.9838$).

3.2.5 Telomerase activity

Telomerase activity was measured in gill tissues of 10 Baltic Sea and 14 Icelandic animals across a wide age range (10-36 year olds from Baltic Sea and, 8-226 year olds from Iceland). In all animals telomere length was also measured as shown above. Most of the investigated animals were females (7 from Baltic Sea and 11 from Iceland) with only a few males (3 from Baltic Sea and 2 from Iceland), or the sex was not determinable (1 from Iceland). The telomerase activity was detected in all ages of both populations but the results yielded extreme variations. Baltic Sea samples had 19.46 - 502203 RT-PCR products/ μg protein and Icelandic samples had 17.53 - 142647 RT-PCR products/ μg protein (Fig 3.12A). This variability suggested that the enzyme stays active throughout the complete investigated lifespan of the animals. Additionally, the enzyme activity in both populations reached comparable levels within the Baltic Sea lifespan (MLSP < 50 years).

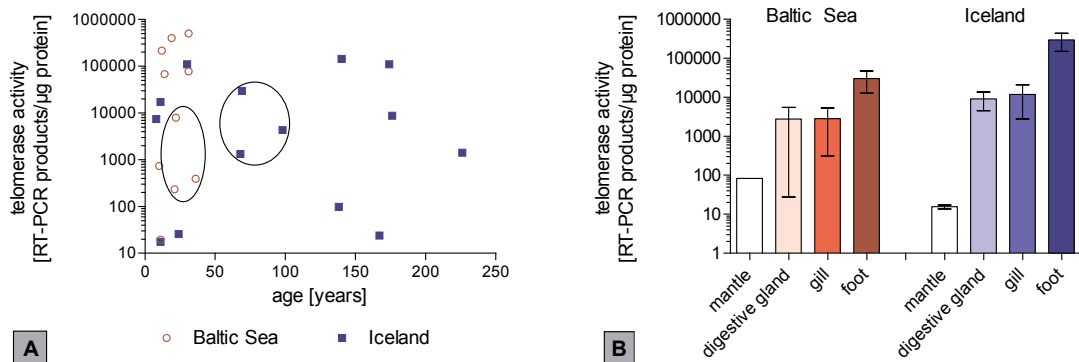


Figure 3.12: Telomerase activity (in no. of extended and amplified template molecules/ μg protein) in gill tissue of both populations (A). Encircled samples were chosen for the tissue catalogue analysis. Mean telomerase activity with SEM in the tissue catalogue of both populations (B) ($n=3$, $n_{BS,mantle}=2$).

The telomerase activity was also measured in tissues with different proliferation rates, such as the gill, mantle, digestive gland, and foot, from both populations. In 83 % of the six investigated animals, the telomerase enzyme was most active in the foot tissue, and in 80 % of the animals the enzyme was least active in the mantle tissue (Fig 3.12B). In both populations the enzyme shows the same trend in activity across different tissues. A correlation of telomerase activity with telomere length

within individuals, was not observable (Fig 3.13).

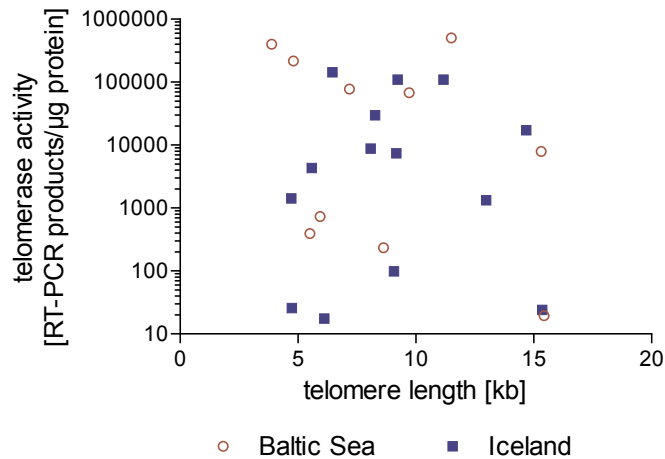


Figure 3.13: Telomerase activity does not correlate with telomere length within individuals. $n_{BS} = 10$; $n_{IC} = 14$.

3.3 Transcriptome analysis

The differences in gene expression between the very long-lived and short-lived population in Iceland and the Baltic Sea, respectively, were assessed by transcriptome sequencing as described in Section 2.3.1. Genes of interest (presented in Tab 3.2), gene richness and different gene expression profiles over age were evaluated to determine possible population specific gene expression behavior that could elucidate the wide gap in MLSP between these two populations. General characteristics of the two population-specific transcriptomes and the final combined transcriptome are listed in Tab 3.1.

Table 3.1: General characteristics and annotation overview of the two population specific (IC_transcriptome for Iceland, BS_transcriptome for the Baltic Sea) and the final combined_transcriptome.

	IC_transcriptome			BS_transcriptome			combined_transcriptome ¹		
	All sequences	100-500 bp	>500 bp	All sequences	100-500 bp	>500 bp	All sequences ¹	100-500 bp	>500 bp
Total no. of contigs	76,835	41,999	34,239	56,294	26,435	29,397	116,375	54,665	60,088
Contigs with matches	16,775	6,466	10,303	14,722	4,839	9,873	23,552	7,119	16,421
Assigned GO-terms	4,835	2,071	2,764	921	264	657	7,798	2,874	4,917
Assigned KEGG-pathways	9,151	3,355	5,795	7178	2,163	5,011	15,041	4,550	10,480
Contigs with InterProScan matches	6,519	2,843	3,676	1,167	343	824	10,377	3,799	6571
Contigs without matches	60060	35,533	23,936	41572	21,596	19,524	92,823	47,546	43,667
% annotated	21.83	15.40	30.09	26.15	18.31	33.59	20.24	13.02	27.33
Total no. of singletons	251,033			174,072			428,249		
Total no. of reads	1,396,146			948,047			3,117,293		
% assembled	82.02			81.64			86.26		

¹: Including contigs covered only by the old transcriptome. Total amount of contigs covered only by the Baltic Sea and Iceland age groups = 109,135.

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Since covering three age groups instead of only two, the Iceland specific database includes more contigs (76,835) compared to the Baltic Sea specific database (56,294). In contrast to the IC_transcriptome, 26 % of the BS_transcriptome is annotated (only 21 % in the IC_transcriptome). 116,375 contigs in the combined_transcriptome could be assembled from >3 million reads, leaving 428,249 singletons. In the final combined RNA seq database 23,552 contigs were annotated (~20 %).

In gene expression analysis from transcriptomes, contig coverage (*i.e.* depth of sequencing) has a great impact on the interpretation of the results. Most contigs in the analyzed combined transcriptome were covered by less than five reads (Fig 3.14).

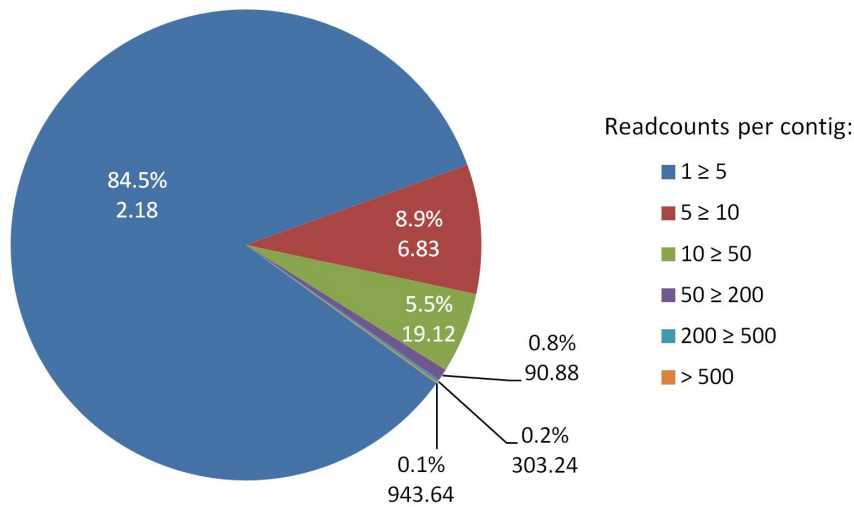


Figure 3.14: Average base coverage in categories of different readcounts per contig. Percent and average of coverage are indicated in respective category sections.

Within the overall transcriptome next to contigs covered by the already published transcriptome [130], 109,135 contigs were created only from the different Iceland and Baltic Sea age groups. The part covered by new contigs was checked for homogenous distribution of the contigs between sampled tissues and age groups of the two populations (Fig 3.15). Notably, more contigs were covered from gill tissue as compared to heart tissue in both populations (21 % vs. 19 % for Baltic Sea, 34 % vs. 26 % for Iceland). The biggest difference was two-fold between contigs from gill (14 %) and heart (7 %) tissues of young Icelandic animals. However, all other groups were represented by 9-11 % of all contigs so that the overall transcriptome consisted of sequences from 10 relatively equally represented groups.

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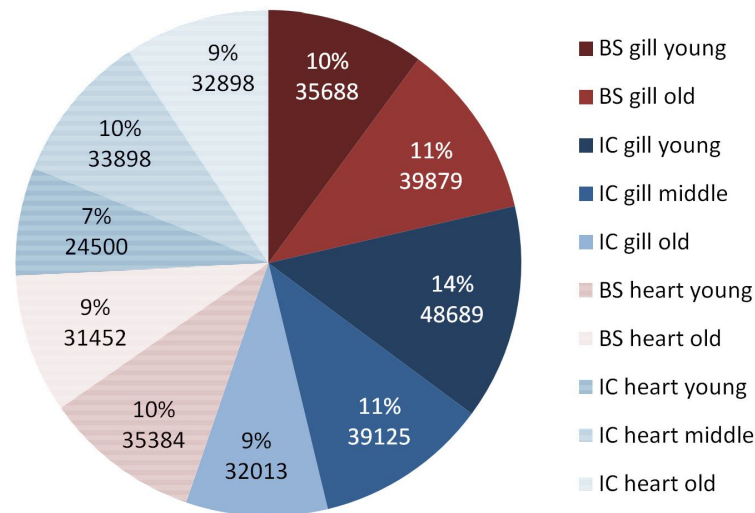


Figure 3.15: Contig distribution in the combined transcriptome between tissues and age groups of the two populations. Percent and number of contigs of each group are stated in the respective sections. (BS=Baltic Sea, IC=Iceland).

3.3.1 Annotation: which genes were found?

As described in 2.3.1.5 the overall transcriptome was searched for relevant genes via keyword-search function in the T-ACE browser. Tab 3.1 shows that only 20.24 % of the combined_transcriptome reflecting both populations was annotated using various databases, including NCBI BLAST, UniProt KB/Swiss-Prot, InterproScan. Tab 3.2 lists the contig IDs in the database combined_transcriptome as well as the corresponding putative gene names, expected and observed domains, E-value, contig length, and number of reads in the contig. Since the analysis of DNA and RNA damage yielded distinct results between Baltic Sea and Icelandic populations (see results in section 3.2.3), further investigation focused on genes that are involved in DNA repair mechanisms. Additionally, genes coding for antioxidants were of interest since reactive oxygen species (ROS) play a role in ageing and these genes could successfully be identified in the transcriptome. Thus, Tab 3.2 shows genes involved in DNA repair, antioxidative processes, ageing-relevant and housekeeper genes investigated via qRT-PCR. A complete list with encountered putative genes in the combined_transcriptome is attached in Appendix p2.

Table 3.2: Genes of interest found in the combined_transcriptome with contig ID, expected domains, observed domains, E-value, contig length, and number of reads in contig. n.a. = not applicable.

Category	Gene name	Contig ID	Domains expected	Domains observed	E-value ¹	Contig length (bp)	No. of reads in contig
DNA repair	FEN1 (flap endonuclease)	16567	H3TH_StructSpec-5'-nucleases, PIN_SF	PIN-SF, H3TH_Str, PIN_S ⁴	0.00	1210	17
	MSH6 (mutS homologue 6) ²	15944	MutS_III, MutS_I, MutS_II, PWWP, TUDOR, MutS_IV, CDC27, P-loop_NTPase	MutS_IV, MutSac ⁵ , p-loop_NTPase ⁴ MutSd, MutS_I, MutS_II ⁴	0.00	1634	18
	NBS1 (nijmegen breakage syndrome)	28936	NBS1_C, BRCT, FHA	FHA ^{4,5} , BRCT ⁵	1E-171	1501	8
	XRCC1 (x-ray repair cross-complementing protein 1)	23004			9E-64	901	11
	XRCC4 (x-ray repair cross-complementing protein 4)	2720	Ebola_NP,PRK13700, XRCC1_N, BRCT	XRCC1_N, 2xBRCT ^{4,5}	1E-153	4401	146
	NHEJ1 (non-homologous end-joining, a.k.a. XLF)	5343	XRCC4	XRCC4 ^{4,5}	3E-69	1453	72
	EXO1 (exonuclease 1)	45649	XLF	XLF ^{4,5}	6E-29	580	5
	p53 (protein 53)	99383	H3TH_StructSpec-5'-nucleases, PIN_SF	PIN_S, H3TH_Stru ⁴	3E-49	446	2
	SIRT6 (sirtuin 6)	27609	p53 tetramer, p53_TAD, p53	p53, p53 tetramer ^{4,5}	7E-87	884	9
	SIRT2 (sirtuin 2)	5121	SIR2	SIR2 ^{4,5}	1E-117	2226	75
Ageing-relevant	SIRT1 (sirtuin 1)	7850	SIR2	SIR2 ^{4,5}	1E-105	2065	46
	CAT (catalase)	9021	SIR2	SIR2 ^{4,5}	1E-174	1916	38
Antioxidants	MnSOD (manganese superoxide dismutase)	1148	PLN02609, catalase_like	Catalase, Catalase_rel ⁴ , catalase_like ⁵	0.00	2459	334
	Cu/ZnSOD (copper/zinc superoxide dismutase)	825	Sod_Fe_N, Sod_Fe_C	Sod_Fe_N, Sod_Fe_C ^{4,5}	1E-101	1669	456
Housekeeper	GAPDH (glyceraldehyde 3-phosphate dehydrogenase)	141	Cu/Zn_Superoxide_Dismutase	SOD_cu ⁴ , Cu-Zn-s ⁵	2E-14	2046	2042
	18S (18S rRNA) ³	54	Gp_dh_C, Gp_dh_N, NADB_Rossmann	Gp_dh_N ^{4,5} , Gp_dh_C ^{4,5} , NADB_Rossmann ⁵	1E-174	2253	3880
	28S (28S rRNA) ³	13	n.a.	n.a.	n.a.	7580	7471
	β -Actin (beta actin)	13	n.a.	n.a.	n.a.	7580	7471
		11	Actin	Actin ^{4,5}	0.00	1580	8684

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- 1: E-values taken from UniProtKB/Swiss-Prot.
- 2: Two contigs were combined for MSH6 revealing a total transcript length of >3 kb.
- 3: 18S and 28S rRNA are covered by the same contig in the DB combined_transcriptome that must include internal transcribed spacers that are situated between 18S and 28S. Primer design was therefore based on contigs found in the already published transcriptome [130].
- 4: Domains observed in PFAM via SMART (smart.embl-heidelberg.com).
- 5: Domains observed in NCBI cDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>)

The low proportion of annotated contigs (20.24 % in Tab 3.1) relates to contigs annotated in any way, meaning nucleotide sequences translated into protein open reading frames (ORFs) that even show certain structures, patterns or protein domains were termed “annotated”. Many revealed domain annotations were misleading. Thus, the results for GO-term or KEGG-pathway analyses were defined to be unreliable and not considered in this work.

3.3.2 Expression profile analysis

Gene expression over age was investigated in the combined_transcriptome along the two age groups sequenced in the Baltic Sea (young, old) and the three age groups in the Iceland population (young, middle, old). Expression profile analyses were based on differently expressed transcripts, or contigs depicted in the transcriptome, between two compared age groups (2.3.1.7). Between the different age groups investigated in gill and heart tissue, the contigs were not significantly differently expressed due to the low sample number (biological replicates with n=4). But the expressed contigs were distinctively different enough to allow for comparison. The number of contigs that were distinctively differently expressed between two analyzed groups is stated in Tab 3.3.

Table 3.3: Number of distinctively differently expressed contigs between analyzed groups (BS=Baltic Sea, IC=Iceland; y=young, m=middle, o=old).

	No. of contigs in IC gill			No. of contigs in IC heart			No. of contigs in BS gill	No. of contigs in BS heart
	y/m	m/o	y/o	y/m	m/o	y/o	y/o	y/o
Compared age groups Distinctively differently expressed contigs	1550	246	1697	649	270	787	306	195

The number of distinctively differently expressed transcript fragments between two age groups in the Baltic Sea is comparable between gill and heart tissues considering

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that there were generally more contigs covered by gill than heart tissues (Fig 3.15). In contrast, in the Iceland population more transcripts were differently expressed in the highly proliferating gill compared to the postmitotic heart tissue. The results also show that the number of distinctively differently expressed contigs among the Iceland population is far less between middle *vs.* old aged individuals in comparison to young *vs.* middle aged and young *vs.* old individuals in both tissues. This trend was confirmed by the analyses of different expression profiles (see Fig 2.8) over age (Tab 3.4). Since most differently expressed contigs did not change between middle and old aged individuals in the Iceland population, this hinted towards possible expression profiles 5 and 8 (plateau up and plateau down, respectively).

A “steady expression” was analyzed by looking for non-distinctively differently expressed contigs. This approach was applied for the analysis of profiles 3, 5, 6, and 8, all of which has to follow the distinct pattern changes in expression. However, analysis of the steady expression profile 9 over the total lifespan was not possible since most transcripts were not distinctively differently expressed over the whole lifespan. Thus, the expression profile analyses revealed the plateau up or down profiles to be the most prevalent (Tab 3.4). Since *A. islandica* is an emerging model organism without the complete genome sequenced, only a few contigs in the profiles are annotated.

Table 3.4: Expression profiles of distinctively differentially expressed contigs from different tissues of IC (Iceland) and BS (Baltic Sea) populations. The total number of contigs and the number of annotated contigs (_{AN}) are listed for each analyzed group. n.a. = not applicable.

Profiles	BS gill	BS gill _{AN}	BS heart	BS heart _{AN}	IC gill	IC gill _{AN}	IC heart	IC heart _{AN}
Hill (1)	n.a.	n.a.	n.a.	n.a.	44	14	31	8
Ditch (2)	n.a.	n.a.	n.a.	n.a.	9	5	4	2
Step up (3)					451	295	147	87
Upstairs (4)	154	68	92	49	6	3	8	2
Plateau up (5)					371	207	168	96
Step down (6)					11	5	14	13
Downstairs (7)	152	67	103	35	6	5	6	4
Plateau down (8)					69	22	52	14

Expression profiles for the Baltic Sea population could only be classified as increasing (profiles 3, 4, 5) or decreasing (profiles 6, 7, 8) over age since only two defined age groups (young and old) were sequenced for this population. Results show that the expression of about equal number of transcripts in the Baltic Sea increase and decrease over age in gill as well as in heart tissues (Tab 3.4). BLAST and/or InterProScan results of annotated contigs of the different expression profiles are listed

in Appendix e2 (Baltic Sea Profiles) and Appendix e3 (Iceland Profiles). In the Iceland population a total number of 1,397 transcripts from gill and heart tissues yielded expression profiles. Relative to the total number of contigs in the combined transcriptome (109,135) and total number of contigs covered by reads of the Iceland population (93,789), this corresponds to approximately 1 % of all contigs. Fig 3.16 visualizes the distribution of the expression profiles amongst the 1 % of Icelandic contigs.

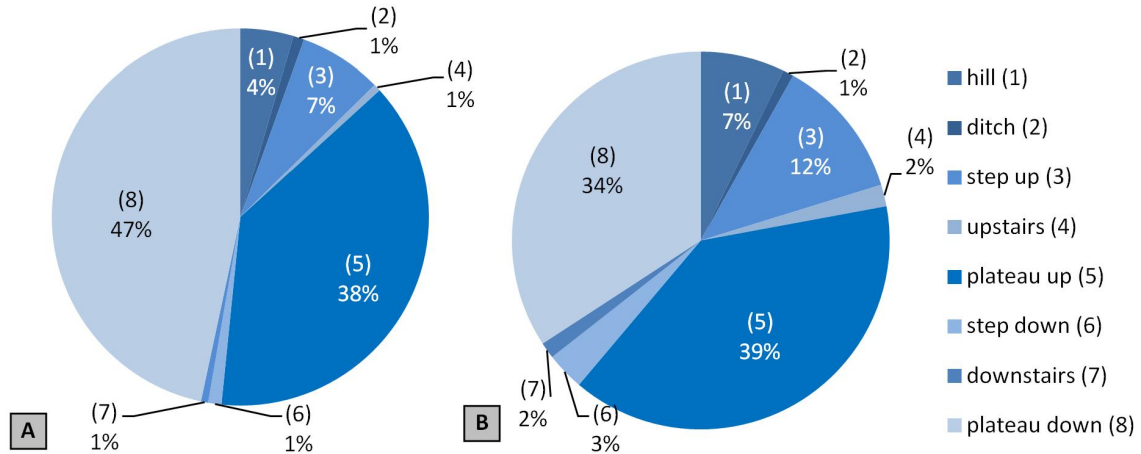


Figure 3.16: Distribution of expression profiles in the Iceland transcriptomes in gill (A) and heart (B) tissues.

The expression profiles are roughly equally distributed in the Iceland transcriptomes of Icelandic gill and heart tissues (Fig 3.16). Clearly, the majority of Icelandic contigs that yielded expression profiles were identified as profile 5 and 8 (85 % in gill and 73 % in heart tissue). Interestingly, one putative gene with a plateau-up expression profile in Iceland gill codes for copper-zinc superoxide dismutase or Cu/ZnSOD. This gene plays a role in the antioxidant defense mechanisms, and was further investigated via qRT-PCR (3.4.2).

3.4 qRT-PCR analysis

The expression analysis of the transcriptome reflected the gene expression in only 4 individuals from three age groups (young, middle, old) in the Iceland and two age groups (young, old) from the Baltic Sea studied *A. islandica* populations. Thus, for a higher resolution of gene expression along the complete lifespan of both populations, a detailed analysis of genes of interest (presented in Tab 3.2) was performed via qRT-PCR.

3.4.1 Sequence validation of selected genes

Since transcript sequences in the transcriptome consist of several assembled reads, it is necessary to verify the correct assembly and hence, the sequence of these transcripts/contigs. Full length primers that at least covered 83 % of the contig (2.3.2.1) were therefore used to sequence the selected contigs by Sanger sequencing. The contigs were sequenced from both directions using forward and reverse primers in order to cover most of their full length. Tab 3.5 shows the genes, sequence length and proportion verified in this work. Other genes of interest were verified by Philipp *et al.* [130] using the same approach.

Table 3.5: Sequenced genes and their verified proportions by Sanger sequencing.

Category	Gene	Sequence verified	percentage
DNA repair	FEN1	1029 of 1029 bp	100 %
	MSH6	1829 of 3038 bp	60.2 %
	NBS1	772 of 772 bp	100 %
	XRCC1	1844 of 3981 bp	46.3 %
	XRCC4	1333 of 1333 bp	100 %
	NHEJ1	481 of 481 bp	100 %
	EXO1	414 of 414 bp	100 %
Ageing-relevant	p53	826 of 826 bp	100 %
	SIRT6	1021 of 1979 bp	51.6 %
	SIRT2	1835 of 1835 bp	100 %
	SIRT1	1803 of 1803 bp	100 %

Most transcripts/contigs <2 kb could be verified in full length since Sanger sequencing yields results for up to 1 kb. Thus, most contigs were verified for 100 % of the primers' product size. The two contigs, MSH6 and XRCC1, both with exceeding 3 kb product size, and the SIRT6 transcript were verified partially. Since parts of each end of the transcripts were sequenced and the PCR product sizes matched the respective contig sizes, the annotation and the contig sequence was assumed as valid. Hence, primer sequences could also be considered as gene specific. Full contig sequences for all investigated putative genes are listed in Appendix e4 (GOI Sequences).

3.4.2 Gene expression over age

The verified genes of interest listed in Tab 3.5 along with three antioxidants (CAT, MnSOD, Cu/ZnSOD) and four housekeeper genes (GAPDH, β -Actin, 18S, 28S) from gill tissues were tested for gene expression analysis using qRT-PCR 2.3.2.7. The Δ CT-method was applied for evaluation of data, and since Normfinder [5] revealed

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best stability value for 28S, this gene was used for all analyses as housekeeper. Outliers were removed using Grubb's test and significances were calculated using the Mann-Whitney test in GraphPad Prism 5.04. The primer efficiencies assessed for these genes are listed in Tab 3.6.

Table 3.6: Primer efficiencies for qRT-PCR primers.

Category	Gene	Primer efficiency
DNA repair	FEN1	1.523
	MSH6	1.640
	NBS1	1.684
	XRCC1	1.931
	XRCC4	1.961
	NHEJ1	1.905
	EXO1	1.861
Ageing relevant	p53	1.795
	SIRT6	1.997
	SIRT2	1.874
	SIRT1	1.916
Antioxidants	CAT	2.259
	MnSOD	2.027
	Cu/ZnSOD	1.880
Housekeeper	28S	2.614
	18S	1.906
	GAPDH	2.005
	β -Actin	1.990

The relative gene expression for Cu/ZnSOD antioxidant gene is shown in Fig 3.17 as an example.

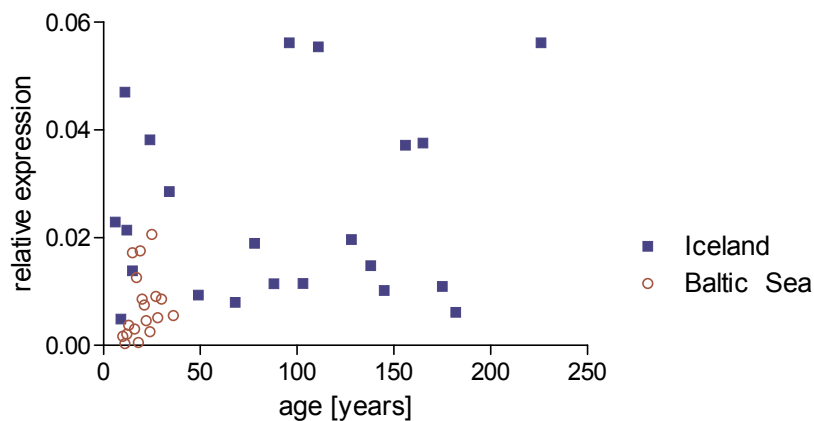


Figure 3.17: Relative gene expression for Cu/ZnSOD in Baltic Sea and Iceland population over age. $n_{BS} = 18$; $n_{IC} = 22$.

The findings from expression profile analyses where the transcript putatively coding for Cu/ZnSOD showed a plateau up profile in the Iceland gill tissue could not be

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verified via qRT-PCR. Similarly, expression profiles over age for other investigated genes of interest were not identified. Tab 3.7 shows the linear models of expression over age for both populations for all investigated genes. The results indicate that there is no recognizable change of gene expression over age (slope $\rightarrow 0$). The corresponding graphs of gene expression over age for both populations are found in Appendix p3.

Table 3.7: Linear models for Iceland (IC) and Baltic Sea (BS) gene expression of investigated genes.

Category	Gene	Icelandic linear model	Baltic Sea linear model
DNA repair	FEN1	$y=-0.004668x+238$	$y=0.0139x+1.032$
	MSH6	$y=8.922e-005x-384.5$	$y=0.001187x-6.171$
	NBS1	$y=2.265e-005x-1227$	$y=0.0005088x-46.89$
	XRCC1	$y=-2.011e-006x+245.4$	$y=1.428e-005x-9.439$
	XRCC4	$y=2.916e-006x-413.7$	$y=1.372e-005x-91.9$
	NHEJ1	$y=4.268e-007x-1697$	$y=4.577e-006x-52.11$
	EXO1	$y=2.458e-007x-276.9$	$y=2.117e-006x-67.78$
Ageing relevant	p53	$y=-4.605e-006x+441.2$	$y=7.991e-005x+5.142$
	SIRT6	$y=-1.723e-007x+287.3$	$y=8.188e-007x-0.6809$
	SIRT2	$y=8.005e-008x-1024$	$y=8.995e-006x+5.056$
	SIRT1	$y=4.769e-007x-1933$	$y=4.693e-006x-85.81$
Antioxidants	Cu/ZnSOD	$y=3.703e-005x-570.6$	$y=0.0001883x-18.33$
	MnSOD	$y=2.660e-007x-1311$	$y=1.874e-005x-10.08$
	CAT	$y=8.631e-008x-613$	$y=2.230e-007x-231.3$

Furthermore, the levels of gene expression in gill tissue were compared between the two populations. Fig 3.18 shows relative expression of investigated genes according to their corresponding categories (DNA repair, ageing-relevant, antioxidants).

Significant differences in expression levels of DNA repair genes XRCC1, EXO1, NHEJ, and FEN1 between the two populations were detected (Fig 3.18A). A significantly higher ($p \leq 0.05$) expression for XRCC1 and EXO1 was detected in gill tissue of the Baltic Sea population. However, FEN1 and NHEJ were expressed significantly higher ($p \leq 0.05$ and $p < 0.01$, respectively) in Icelandic gill tissue. These genes play a role in the DNA repair pathway Non-Homologous End-Joining (NHEJ), which is the predominant double-strand break repair pathway in metazoans [90]. XRCC1 and FEN1 are also core proteins in the base excision repair (BER) pathway responsible for the correctness of altered single nucleotides. The presumably ageing-relevant genes SIRT1 (involved in transcription regulation) and p53 show significantly higher ($p < 0.01$ and $p \leq 0.05$, respectively) expression in gill tissue of the Iceland compared to Baltic Sea animals (Fig 3.18B). The antioxidant, MnSOD is significantly higher ($p \leq 0.05$) expressed in gill tissue of the Baltic Sea population, but the strongest significance ($p < 0.0001$) for the higher expression level was observed for the antioxidant gene Cu/ZnSOD in the Iceland population (Fig 3.18C).

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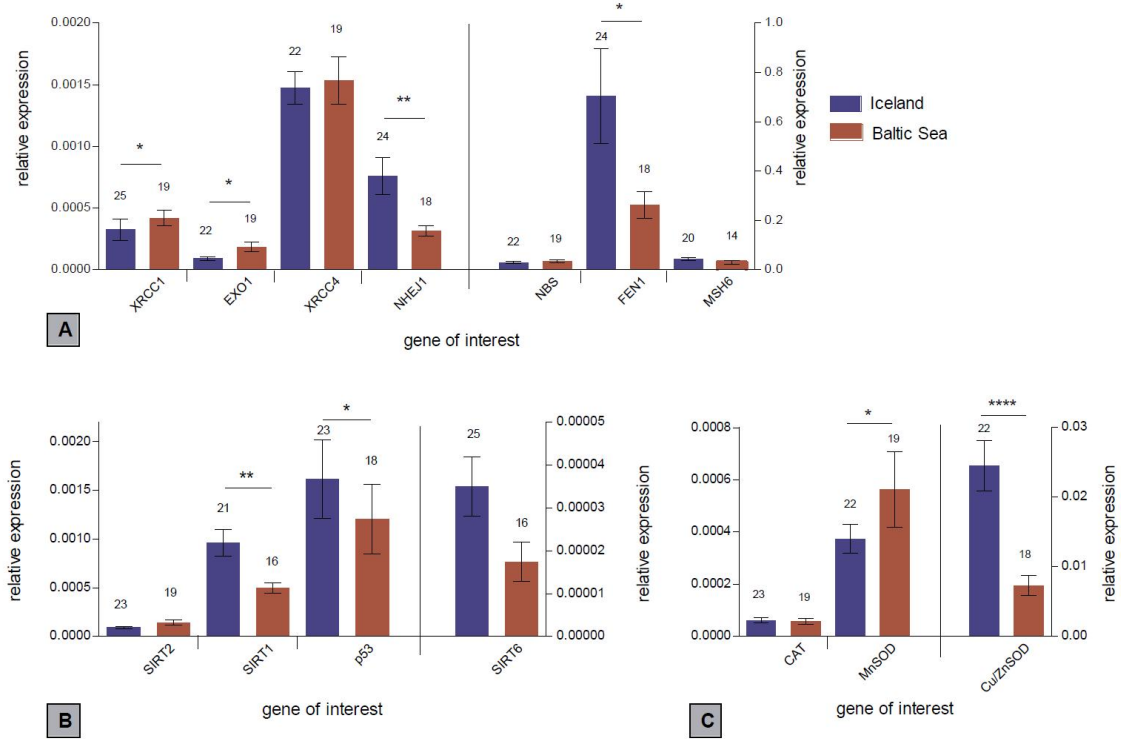


Figure 3.18: Levels of gene expression of (A) DNA repair genes, (B) ageing-relevant genes, and (C) antioxidants in gill tissue of Baltic Sea and Iceland animals. Asterisks indicate significance ($=p \leq 0.05$, $**=p < 0.01$, $***=p < 0.0001$). Numbers above columns indicate the number of analyzed samples. The vertical lines separate data plotted on the left y-axis from data plotted on the right y-axis.

Taken together, waste accumulation or a significant change in investigated ageing parameters or gene expression of genes of interest over age was not revealed, except for the oxidation of nucleotides that dramatically changed over age and significantly differed between both populations. Levels of lipid peroxidation were significantly higher in the Iceland population and gene expression profiles over age revealed distinct differences between young individuals compared to middle and old aged ones. In the following (Discussion), all results from the population characterization, ageing marker-, transcriptome-, and qRT-PCR expression analyses will be taken together and discussed to reveal concluding findings of this work.

4 Discussion

In the presented work selected individuals from a short-lived *Arctica islandica* population from the Baltic Sea (MLSP ~ 40 years) have been compared to the longest-lived population from Iceland (MLSP >400 years) with respect to physiological damage, molecular ageing markers, as well as gene expression behaviour to investigate the different ageing processes of the two populations. Environmental and population characteristics are also provided and discussed below since these are germane for the understanding of the distinct ageing processes of the two populations. The outcomes of each studied parameter are discussed below and brought into context with each other in the Conclusion and Outlook section (Chapter 5).

4.1 Distinct population characteristics

The phenotypes of the investigated populations from the short-lived Baltic Sea and long-lived Iceland are very distinct [70] (personal observations). However, the growth curves based on height (referred to as LSG or line of strongest growth) and age (determined by annual rings) of these two sampled populations are in concordance with each other and the findings by Witbaard *et al.* [191], who found distinct growth rates of different populations but similarly shaped growth curves. The two growth curves of the investigated populations in this study differ only when the Baltic Sea growth curve terminates at 36 years of age and Iceland continues until 226 years. The shape of both growth curves resemble the ones described in previous studies with a steep, relatively linear increase for the first 30-40 years and a diminishing growth afterwards [18, 161, 70]. Compared to other bivalves, however, *A. islandica* exhibits a relatively slow growth [161]. Clusters of high offspring recruitment lasting for approx. 30-40 years and gaps of the same size (30-40 years) between those clusters, so-called “recruitment gaps”, can be observed in the Iceland growth curve as previously stated by Murawski *et al.* [110], Steingrímsson & Thorarínssdóttir [158], and Powell & Mann [133]. There are a number of studies that investigated growth [161], enzyme activity [2], and maturation [170] of *A. islandica* indicating that during

the first nearly 40 years the animals undergo development. So, it is interesting that the time span of this development coincides with the length of the recruitment gaps observed in the lifespan of the sampled Iceland *A. islandica* population in this study. Based on the above stated findings and the oldest sexually-intermediate individual found by Thorarinsdottir & Steingrímsson [170] at 43 years of age, I speculate that even though *A. islandica* can generally mature sexually at younger ages, for example, as young as 5 years [138], reproduction scarcely takes place until around 30-40 years of age. With increasing size, the reproductive output of older individuals also increases due to larger gonads of bigger animals [128]. Interestingly, in the Baltic Sea population, which lives up to approx. 40 years of age, a strong recruitment during the past decades has been observed by Zettler *et al.* [198]. Other studies propose that in the Baltic Sea the population structure differs completely from the Atlantic Ocean populations, in which small size classes are almost absent [110, 158, 198]. Our own observation during the sampling progress, however, revealed a large number of small individuals in the Iceland population. The evaluation of the smaller samples – a project in progress by the cooperation partner, Max Planck Institute (MPI) for Demographic Research, Rostock – will yield further detailed results on the demographic distributions of the two populations.

For humans and several other mammals [190, 166, 137, 193] and also Crustaceae [61, 184], differential mortality rates between sexes are known. A clear shift in sex ratio towards females could be observed in the Iceland population at older ages. This has also been indicated by Thorarinsdottir & Steingrímsson [170] who observed that younger size classes in the Iceland *A. islandica* population were dominated by males and older size classes by females. Within the Baltic Sea lifespan sexes were evenly distributed in both populations; however, the very low sample numbers at younger ages in the Iceland population, might be a sampling artifact that affects sex distribution in this age group. More data are being evaluated by the MPI, Rostock, to confirm these findings, especially for younger age classes. The analyses of the large samples (179 individuals from Iceland, 160 individuals from Baltic Sea) of the two very distinct populations confirmed previous studies regarding growth of *A. islandica* [191, 161], but also led to new findings such as the population structure in the Atlantic ocean Northeast of Iceland showing many small individuals (age determination in progress by MPI, Rostock) and sex distribution in the Iceland population that has only been scarcely investigated so far [170].

In addition, environmental factors that might have an influence on the animals growth, reproduction, and other investigated parameters that affect ageing, differ a lot between the two habitats and need to be mentioned here. Tab 4.1 shows data from Basova *et al.* [16] on the extreme environmental differences between the two

investigated populations.

Table 4.1: Environmental data for the two locations of sampled populations (data taken from Basova *et al.* [16]).

Population	Location	Mean annual salinity (S), ppt	Amplitude of seasonal S variation, ppt	Mean annual temp. (T), °C	Amplitude of seasonal variation in T, °C	Recorded MLSP, years
Iceland	66°02N 14°51W	35	0.4	4.1	6.2	>400
Baltic Sea	54°32N 10°42E	20-25	4.8	7.3	9.3	<50

Limited high oxygen water and high salinity water inflow from the North Sea into the Baltic Sea (Fig 4.1A) result in limited oxygen supply and stratification of the water column preventing vertical mixing and the transport of more oxygenated waters to the bottom [40]. Recurring, fluctuating hypoxic conditions – especially close to and in the sediment, where *A. islandica* lives, – force the animals to constantly adapt their metabolic behaviour to their environment. In the case of oxygen absence or low oxygen availability, *A. islandica* can undergo metabolic rate depression (MRD) a state comparable with hibernation [168]. Philipp *et al.*, [130] speculated that reactive oxygen species (ROS) generation during the re-oxygenation process after MRD takes place so that a repetitive switch from normoxic to hypoxic/anoxic conditions exposes the Baltic Sea animals to a non-manageable dose of ROS which damages their physiological constitution leading to a shortened lifespan. In contrast, upwelling currents around Iceland constantly supply animals with oxygen rich and high salinity water (Fig 4.1B) that serve for a high biomass production and therefore, food supply for *A. islandica* [9]. The investigation of mass specific respiration rates of *A. islandica* from five different sites showed no change in respiration rate over age and no population specific metabolic rate [16, 19]. Lowest metabolic rates were found in the warm-adapted German Bight and the cold-adapted Iceland population indicating no temperature-dependent metabolic rates. This observation suggests a more economical metabolic behavior of the long-lived Iceland *A. islandica* population where conditions are more stable compared to the Baltic Sea environment [16] and animals are not repeatedly forced to undergo MRD.

Individuals exceeding the age of 300 years are very scarcely observed in Iceland (two individuals reported so far), whereas animals around 200 years are more frequently found. However, the age of 40 years is generally accepted as MLSP in the Baltic Sea. In this study, at least one 36 years old Baltic Sea animal and one 226 years old Iceland animal sufficiently represent the corresponding MLSPs for these populations since these sampled animals correspond to 90 % of the Baltic Sea MLSP and 75 %

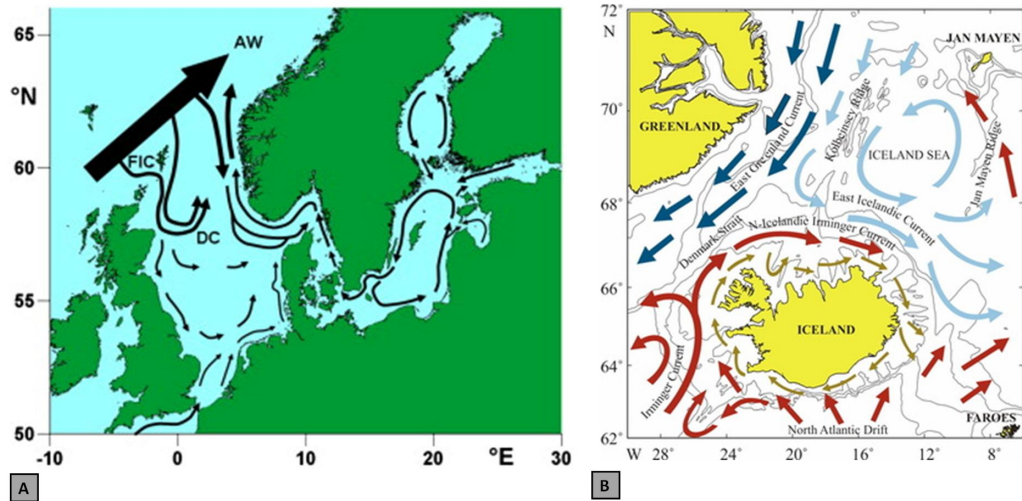


Figure 4.1: Surface circulation in the North Sea and Baltic Sea (A) based on Elken and Matthäus [14] and on Svendsen *et al.* [165], respectively, and currents around Iceland (B) (modified from Astthorsson *et al.*) [9]. AW = Atlantic Water, FIC= Fair Isles Current, DC = Dooley Current. The arrow thickness in (A) is indicative for the relative magnitude of the volume transport. (A) taken from Dippner *et al.* [47].

of the Iceland MLSP when considering 300 years as MLSP. Hence, these sampled animals provided a solid base for the ageing studies.

4.2 Analysis of ageing markers

Many studies have provided results on ageing markers in a great number of different species [175, 32, 13, 156, 72, 31]. However, thus far, no study has been conducted covering such a wide range of ageing markers across such a long lifespan in the longest-lived non-colonial animal known to date. In *A. islandica*, Strahl *et al.* [161] measured lipofuscin, a fluorescent age marker pigment, and protein oxidation by quantifying protein carbonyls in the Iceland population. Several oxidative stress defense parameters, that give information on the oxidative stress response, such as the ROS-scavengers catalase (CAT), superoxide dismutase (SOD), the redox potential of glutathione as a ratio of oxidized and reduced glutathione (GSSG:GSH) over age, and the mitochondrial activity marker citrate synthase (CS) were investigated by Abele *et al.* in the Iceland population of the ocean quahog [2]. In the work presented here protein carbonyls and stability, lipid peroxidation, nucleic acid damage (DNA and RNA oxidation) and telomere maintenance (telomere length, telomerase activity) were measured in the short-lived Baltic Sea and the long-lived Iceland population of *A. islandica* in a more comprehensive study.

4.2.1 Cellular damage

In his article titled “Cats, ‘Rats,’ and Bats: Comparative Biology of Aging in the 21st Century” Steve Austad [13] states that a “provocative link between integrity of the proteome and longevity” underlines the disposable soma theory. This link is supported by several comparative mammalian studies which investigated protein oxidation and stability [123, 142]. Their comparative observations, of lower protein damage in long-lived species compared to shorter-lived species are confirmed by Strahl *et al.* [161] who found a lower level of protein carbonyls in the long-lived *A. islandica* bivalve compared to shorter-lived and faster growing scallops or clams. These inter-species results are, however, contradictory to intra-species findings by Strahl *et al.* [161] who also investigated protein oxidation over age in the long-lived population of *A. islandica* from Iceland and could not observe an increase of protein carbonyls from young to old aged individuals. A significant increase in oxidatively damaged protein levels with age could neither be detected in *A. islandica* nor in the long-lived rodent the naked mole rat [31]. Besides protein oxidation in one population, protein oxidation, protein stability and lipid peroxidation were investigated in the short-lived Baltic Sea as well as the long-lived Iceland population of the ocean quahog over age. All three measured parameters stayed stable over age. Hence, an accumulation of the damage parameters with years could not be detected in 11-35 year old Baltic Sea individuals and 11-226 year old Iceland individuals. Levels of protein oxidation and protein stability – soluble and insoluble proteins – were equal in both populations.

The two very different environmental conditions of the populations from both habitats either do not play a role for the maintenance of the proteome or adapted repair mechanisms help to overcome environmental constraints so that the proteome equally functions in both populations and is thus not responsible for the observed discrepancy in MLSPs. The observed significantly higher level of lipid peroxidation in the long-lived Iceland population compared to the short-lived Baltic Sea population corroborates findings from Andziak & Buffenstein [6] and Andziak *et al.* [7] who found higher levels of lipid peroxidation in the long-lived rodent, the naked mole rat, compared to short-lived mice. Measurements of lipid peroxidation in the past have mainly been based on quantification of malondialdehyde (MDA) [163, 151, 78] which lacks specificity, whereas the quantification of F₂-isoprostanes derived from the free radical oxidation of arachidonic acid is a more exact biomarker for lipid peroxidation [13]. Abele *et al.* [1] who investigated several clam species from different environments postulated that lipid composition in cold adapted species or populations might differ from animals living in more moderate temperate environments in a way that make

them more prone to lipid peroxidation. Higher levels of polyunsaturated fatty acids at low temperatures may elevate the inherent susceptibility to lipid peroxidation [42]. Findings from the presented ageing-marker investigations presented in this work may corroborate findings from Lieber & Karanjawala [90] who observed that genes involved in proteome maintenance and diseases affecting the proteome have not been associated with ageing so far. However, since no significant increase of investigated damage parameters over age have been detected in *A. islandica* neither in this nor in previous studies [161], the maintenance of an intact proteome might, hence, be of high importance for longevity.

4.2.2 Molecular damage

The major source of endogenous DNA damage is ROS [92, 23]. The oxidation of nucleotides in the cell nucleus and presumably also in mitochondria (at least for mammals) primarily leads to the formation of oxidized guanosine, 8-oxoGuo, which is particularly sensitive to ROS due to its low redox potential [115]. These base pair alterations mainly undergo the base excision repair pathway (BER) [98]. However, the oxidation of nucleotides can also result in double strand breaks (DSBs) [134]. 8-oxoGuo is highly mutagenic and may lead to miscoding of G:C to A:T transversion mutations [57]. High levels of 8-oxoGuo can be found in patients with malignancies and antioxidant repair enzymes may be altered in cancers [41, 76, 94]. The oxidation of nucleotides was the only investigated parameter in this work that increased with age in both populations. An increase of DNA oxidation with age has also been observed in several other organisms, such as rodents, *Drosophila*, and in muscle tissue of humans [62, 178]. Hamilton *et al.* [62] actually detected an increase of DNA oxidation between 21-370 % over 18 months lifespan in several tissues of rodents. In gill tissue of Iceland *A. islandica* DNA oxidation levels between 6 and 226 years increased by 6.73 % per year and in the Baltic Sea DNA oxidation levels between 10-36 year old animals increased by 9.24 % per year. Hence, the results in the presented study revealed a significantly slower accumulation of oxidative damage to nucleotides in *A. islandica* compared to other species which was, however, significantly faster in the Baltic Sea compared to Iceland. Additionally, a higher level of DNA damage in the Baltic Sea population was detected. The higher susceptibility of the Baltic Sea population to nucleotide oxidation might be derived from the less stable and warmer environment in the Baltic Sea where events of hypoxia and anoxia are quite frequent and force the animals to continuously adapt their metabolic behavior to the given conditions (e.g. by undergoing metabolic rate depression).

Interestingly, with regard to the relative lifespan of both populations ($MLSP_{IC}$

= 300 years, $MLSP_{BS} = 40$ years), slopes of DNA and RNA damage accumulation were equal (Fig 3.10). Based on the nucleotide damage process observed in both populations over their relative lifespan, it begs the question whether *A. islandica* populations reach the maximum lifespan that they are able to reach. The comparable increase in nucleotide damage over relative lifespan in both populations might point towards a certain bearable damage level that limits the populations' lifespans to their respective MLSP.

The investigation of proteins and lipids did not reveal a damage accumulation over age and hence, seem to be under control, *i.e.* damage is sufficiently prevented or repaired. Higher levels of lipid peroxidation in the long-lived Iceland population of *A. islandica* apparently do not play a role in attaining a long lifespan. However, unlike proteins, lipids and RNA that can be replaced, damage to DNA must be repaired [90]. The results show that oxidative damage to nucleotides cannot sufficiently be prevented or repaired in order not to accumulate with age. This accumulative parameter, rising significantly faster over age in the shorter-lived Baltic Sea *A. islandica* population compared to the longer-lived Iceland population, might therefore play a crucial role in the ageing process of this bivalve mollusk. The core proteins in the BER pathway, that primarily repairs altered nucleotides, are APE1, POL β , FEN1, and the DNA LIG1 or LIG3/XRCC1 complex [98]. The main pathway to repair DSBs, that may also arise from oxidative damage to nucleotides, is Non-homologous end-joining (NHEJ) [167] with the following core proteins: Ku, DNA PKcs, POL β , XRCC4/DNA LIG4/XLF [90, 98, 167]. A selection of genes coding for some of these core proteins could be identified in the generated RNAseq database and were further investigated via qRT-PCR (Section 4.4.2).

4.2.3 Telomere maintenance in *A. islandica*

In both *A. islandica* populations, a high heterogeneity of telomere length and telomerase activity was observed at all ages, which supports Philipp & Abele [128] who speculated that animals with extreme long life may have evolved some telomerase independent mechanisms to prevent telomere shortening. The decrease of telomere length with age previously seen in gill tissues of scallops [48] was not observed in highly-proliferating gill tissues of the short- and long-lived *A. islandica* populations. In contrast, stability of telomere length over a wide age range was detected.

Measurements of telomerase activity show an analogous picture to telomere length examinations. The high and low enzyme activities detected in individuals at any age in both populations indicated that telomerase expression was not silenced in

adult tissue as known to occur in humans. Preferential telomerase activity on short telomeres previously observed by Ouellette *et al.* [120] in human tissue was not seen in *A. islandica* where telomerase activity was high or low regardless of telomere length (Fig 3.13). A sustained expression of telomerase could underlie other functions of the enzyme (e.g. protection of mitochondria, inhibition of apoptosis, enhancement of DNA repair, promotion of cell growth and stem cell proliferation) [26] so that a correlation with telomere length cannot be observed. Telomerase was detected in different *A. islandica* tissues (gill, mantle, digestive gland, foot), with lowest and highest activity measured in mantle and in foot, respectively. Additionally, the same relative activity trends were observed in both populations (Fig 3.12B). Previous studies also detected telomerase activity in diverse tissues of several marine organisms with indeterminable growth along all investigated ages [81, 82, 121]. The high variation in telomere dynamics - both telomere length and telomerase activity - in the different age classes may be due either to environmental conditions or individual properties. Personal observations (E.R.R. Philipp) showed a reduction in *A. islandica* foot size after long exposure to anoxia. As a hypothesis to explain this observation, the foot might serve as glycogen storage tissue, which diminishes under debilitating conditions, and highly proliferates and expresses telomerase needed for tissue regeneration. This is supported by Belair *et al.* [20] who actually demonstrated that telomerase activity is a marker for cell proliferation rather than for malignant transformation. And even though telomerase activity has been observed in >85 % of human cancers [79, 83], in *A. islandica*, where only one cell transformation has been detected so far [124], telomerase is active throughout all investigated ages and does not seem to trigger cancer formation. However, since high levels of oxidative damage to nucleotides was found in both populations of *A. islandica* in the presented study and in human cancers [76, 94] and DNA repair activity seems to be reduced in cancer patients [41], an investigation of genes coding for DNA repair core proteins is of high interest. Expression of DNA repair genes that could also explain observed differences in DNA and RNA oxidative damage between the two investigated populations (see 4.2.2) were, thus, identified in the established RNAseq database and analyzed via qRT-PCR (section 4.4.2).

Furthermore, cross-sectional-analyses are limited in their interpretations about ageing marker accumulation or decline over age and life stages of a single individual since realtime tracking over an individual lifetime is not performed. Thus, there is no evidence about investigated damage parameters, such as protein carbonyls, lipid peroxidation, nucleotide oxidation or telomere dynamics over age or over life stages between earlier life and adulthood of a single *A. islandica* individual. Nevertheless, significant differences in levels of lipid peroxidation and DNA oxidation between

the investigated short-lived and long-lived *A. islandica* populations and significant increases of nucleotide oxidation (RNA and DNA) in both populations strongly indicate an ageing-relevant role of these factors.

4.3 Transcriptome analyses

The generation of the RNAseq database of this non-model organism was highly complicated by the missing genome sequence. Additionally, sequence complexity was significantly increased at first with the inclusion of 20 different individual animals (see setup Fig 2.6), and subsequently two different tissues were taken from each animal and multiple animals were sampled to represent different age groups from two very distinct populations. The generation of the population-specific transcriptomes was one helpful step to reduce sequence complexity, which was reflected by the percentage of reads that could be assembled into contigs (Tab 3.1). Approx. 82 % of all reads could be assembled in the population-specific transcriptomes whereas an increase of reads that could be assembled in the final combined transcriptome could be observed (86 %). When looking at the sequencing depth, however, the average base coverage was only 2.18 in 84.5 % of all contigs (Fig 3.14). This can be due to several reasons. As mentioned above, very complex sequences hampered the assembly process. One population or individual might have a higher mutation rate as the others leading to mismatching reads. The sampling of wild marine animals may allow for co-purification of foreign RNA that contaminates the sample and further increases complexity of the sequences. This possibility, however, was minimized since blast results of investigated genes did not lead to species such as bacteria and algae. Another explanation for the low coverage is that the *A. islandica* transcriptome might be unexpectedly big, and sequences generated by 2 $\frac{1}{2}$ picotiter plates (*i.e.* \sim 2.5 million reads) could not cover the complete transcriptome. Since the genome of *A. islandica* is still unknown, an estimate of transcriptome size is difficult to obtain. However, other bivalve transcriptomes generated at our Institute of Clinical Molecular Biology (IKMB) (*M. edulis* with approx. 74,000 contigs from ca. 2.4 million reads [129], *L. eliptica* with approx. 42,000 contigs from ca. 1.3 million reads [74]) were much smaller. For the final combined transcriptome used in this study, more than 3 million reads were assembled into approx. 116,000 contigs, whereas in the population specific transcriptomes a higher ratio of contigs/reads could be assembled with the IC_transcriptome resulting in approx. 77,000 contigs from 1.4 million reads and the BS_transcriptome resulting in approx. 56,000 contigs from 948,000 reads. This is a good reduction in sequence complexity. However, since the average base coverage in the final RNAseq database is very low, further assembly

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steps could still improve the transcriptome quality, *i.e.* coverage. For instance, some investigated genes could be identified by more than one contig (e.g. MSH6, MnSOD). Contig coverage in long contigs is sometimes very heterogeneous across the whole contig length indicating an inconsistent assembly of single domains (isoforms) (Fig 4.2). It is possible, that reads which highly cover a domain and are adjacent to regions with a rather low coverage, are simply missing in the assembly of other contigs.

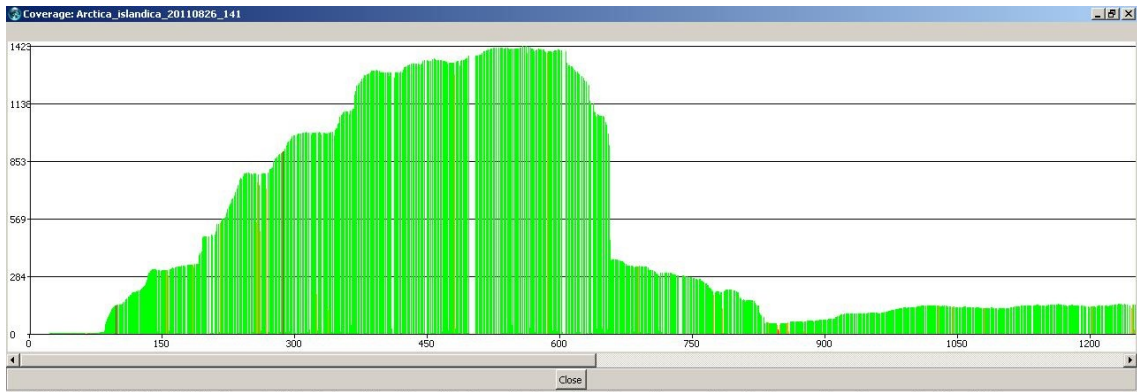


Figure 4.2: Contig coverage on y-axis over contig length of Cu/ZnSOD on x-axis demonstrating high heterogeneity in single base pair coverage across complete contig length (screenshot from combined_transcriptome in T-ACE browser).

For a *de novo* assembly long read length provided by 454 pyrosequencing technology from Roche (400-600 bp at the time of sequencing), is appropriate, especially for transcriptome generation as reads spanning more than one exon are important for a correct assembly of transcribed areas. For instance, common protein domains occurring in multiple genes and high repetitive sequence regions are more likely to be correctly assembled with longer than with shorter reads. 454 pyrosequencing has previously been successfully used for *de novo* assemblies of other non-model organisms [164, 140]. Hence, other next-generation sequencing (NGS) technologies that are also available at the IKMB (Illumina Solexa, SOLiD sequencing) but yield comparatively short read length (50-200 bp) were not considered. The “Gold Standard” of sequencing when considering reliability and sequence length remains to be the Sanger sequencing method. Due to the chemicals this quite expensive method is, however, not applicable for high throughput sequencing needed for transcriptome generation. The 454 pyrosequencing method was therefore the technique of choice. This method relies on chemiluminescence detection, but requires real-time detection as light fades quickly and accurate quantification of the light amplitude is important to capture and assess multiple base incorporations from homopolymers. The detection of homopolymers is one drawback in this technology. The technique also incorporates the four bases A, T, G, and C in separate reactions. Hence, the lack of nucleotide

competition in each reaction increases the polymerase's misincorporation rate [4].

The investigation of expression profiles across covered lifespan, however, can be justified by a relatively equal representation of the different age groups, tissues and populations by the transcriptome (Fig. 3.15). Prior to expression profile analysis a normalization on contig length was performed (RPKM-value 2.3.1.7.1), so that the heterogeneity in average base pair coverage is the same for all contigs and independent from origin of the reads and hence, this artifact does not affect the analysis of expression profiles over age.

4.3.1 Annotation

Sequence knowledge of marine bivalves, especially *A. islandica* has been very scarce so far. The publication of *A. islandica* sequences from a transcriptome generated at the IKMB in 2009 [130] largely enhanced this knowledge, but it can even be improved with the generation of the complex RNAseq database used in this work. Additional work in this area will also provide more annotation of sequences from bivalves. Herein lies the drawback of working with a non-model organism. As no genome is published and annotation of sequences is very scarce, the choices of genes of interest for further detailed study are very limited. This current lack of annotation could also be seen by the analyses of GO- and KEGG-terms which did not lead to valuable results. As animals did not undergo certain treatments, such as temperature stress or oxygen depletion, except for the naturally occurring process of ageing, identification and further analyses of relevant biological pathways (e.g. immune response, antioxidant defense) could not be readily performed. Since results of the oxidative damage markers in this study revealed a significant difference in nucleotide oxidation between the two populations (section 3.2.3), the attention was, of course, drawn towards DNA repair mechanisms, antioxidant defense and commonly discussed ageing-relevant genes. However, most of these genes generally play a role in cell maintenance and metabolism and are continuously expressed so that selected genes of interest for qRT-PCR analysis were previously screened in the literature to be stress regulated as discussed in section 4.4.1.

4.3.2 Expression profiles

Gene expression analysis by transcriptional profiling referred to as digital gene expression profiling, is suitable to infer relative gene expression levels [45]. The change in gene expression over age was analyzed in different age groups that were

represented in the transcriptome database. A wide age range of the Icelandic population was covered here (11-226 years) in three different age groups represented by four young, four middle, and four old aged female individuals. The short lifespan of the Baltic Sea animals (10-36 years in the sample) was depicted in the RNAseq database by only two age groups of four young and old female animals in each group. Thus, the change in gene expression in the Baltic Sea could only be determined as increasing or decreasing whereas more detailed expression profiles (see Fig 2.8) could be investigated for the Iceland population.

Each NGS technology has its specific sequencing bias. However, in general, for RNAseq it is often crucial to correct for gene length when calculating gene signals [45]. This has been considered when calculating the RPKM-value (2.3.1.7.1). It also has to be kept in mind that all expression profile analyses were based on distinctively differently expressed contigs since the number of individuals in each age group ($n=4$) was too low to reveal significant differences after correction of the p-values for multiple testing by Benjamini-Hochberg. Hence, only for approx. 1 % of all contigs covered by Iceland animals distinctly changing expression profiles over age could be determined. In contrast to the Baltic Sea population, more detailed profiles could be identified in the Iceland population with three age groups represented in the transcriptome (Fig 2.6). Here, the majority of the contigs displaying an expression pattern showed plateau-up (profile 5) and plateau-down (profile 8) profiles (85 % in gill, 73 % in heart tissue). Even though overall this represents only a very small amount of the transcripts, this finding goes in line with other results from Abele *et al.* [2] in which the investigated parameters (glutathione peroxidase, citrate synthase, catalase, superoxide dismutase) showed similar activity patterns over age with higher enzyme activities at younger ages, then a rapidly declining and a flattening activity curve for older ages. This indicated that young aged *A. islandica* individuals – 12 years of age in the transcriptome database – still undergo high changes in physiology. Young animals showed faster growth and also higher activity rates for several enzymes [2] up to an age around 25-40 years, which coincides with the oldest sexually intermediate animal (43 years of age) found by Thorarinsdottir & Steingrimsdottir [170] and our findings regarding the recruitment gaps in the observed growth curve. Since the time in which physiological changes still occur comply with the MLSP of the Baltic Sea *A. islandica* population, different life strategies might have evolved in the two distinct populations. Additionally, according to the antagonistic pleiotropy theory, factors that are beneficial to obtain a long lifespan might rather be selected for in the longer-lived Iceland population since reproductive output in older/larger animals is much higher than in younger/smaller ones.

These results indicate that ageing is very unlikely due to single genes, but rather to

complete pathways or repair/defense mechanisms. Studying pathways and networks as integrated systems are therefore necessary to help to elucidate causes of ageing. So-called functional enrichment analyses that incorporate gene annotation to identify enriched functions and processes are however, only feasible for organisms with available and sufficient annotation information. Although this is not the case with *A. islandica*, the pure readout data can be used in network analyses to identify co-expressed genes that may be under similar regulation, e.g. as shown by Hüning *et al.* [73]. These analyses, admittedly, make it difficult to determine the causal hierarchy of two co-expressed genes [45, 93]. Nevertheless, a reliable analysis of expression levels is much enhanced by deeper sequencing. This can be achieved by sequencing with a higher coverage rate by Illumina or SOLiD sequencing technologies that both produce roughly 40-50 times more sequence data per run in comparison to 454 pyrosequencing [45]. A quantification of expression levels of exons or specific alleles is thus, more reliable when using different sequencing platforms than 454.

4.4 Detailed expression analysis

The present transcriptome conditions did not allow for a reliable analysis of gene expression levels with a high resolution over age in the investigated *A. islandica* populations. Selected genes of interest described in the following were therefore analyzed via qRT-PCR along the whole lifespan covered by the sampled animals.

4.4.1 Selection of genes of interest

Genes relevant to ageing and playing a role in DNA repair and antioxidant defense mechanism were in the focus of interest in the study presented here. The transcriptome was scanned via keyword-search for known ageing-relevant genes and those proposed by Kuningas *et al.*, Christensen *et al.*, Guarente & Kenyon, Nebel *et al.*, Feng *et al.* [85, 39, 59, 113, 50]. In addition to its function in tumor suppression, cell cycle regulation and apoptosis, p53 has also been found to be involved in ROS and DNA damage response. In a context-dependending manner, p53 has been shown to promote or prevent ageing [50] and was therefore selected as a gene of interest. Sirtuins (SIR), originally identified for cellular regulation in yeast as “Silent mating-type Information Regulation 2”, generally exert histone deacetylase or mono-ribosyltransferase activity and thus regulate biological pathways via transcription regulation. For instance, by the regulation of p53 via deacetylation or through empirical experiments with *C. elegans*, SIR2 genes have also been associated with senescence.

Detailed expression analysis via qRT-PCR was thus performed on selected, reliably identified ($E\text{-value} > 1E-100$) SIR2 genes (SIRT1, SIRT2, SIRT6). According to the free radical theory of ageing by Harmann [65] the oxidative stress response plays an important role in the prevention of ageing. This theory, *A. islandica*'s ability to undergo prolonged periods of metabolic rate depression (MRD) [168], and recent studies of the Baltic Sea population's antioxidant defense mechanisms that revealed a different expression behavior compared to other longer-lived populations [185, 130] were reasons to select genes coding for the antioxidants catalase (CAT), manganese superoxide dismutase (MnSOD), and copper/zinc superoxide dismutase (Cu/ZnSOD) for qRT-PCR analyses. The sequences of the antioxidant defense genes have been published by Philipp *et al.* [130], but sequences for ageing-relevant and DNA repair genes were verified by Sanger sequencing for this work. DNA repair genes were investigated since DNA damage levels and accumulation over age were very distinct between the short-lived Baltic Sea and the long-lived Iceland populations (see 3.2.3). Because most DNA repair genes are ubiquitously expressed and present in animals at any age, only genes which had been searched for via the keyword "DNA repair" and previously been shown to be stress regulated or ageing-relevant (according to literature discussed below) were considered for the qRT-PCR analysis. All selected DNA repair genes are present in each cell at any time since they are needed for the maintenance of the genome. Mistakes by the genome replication machinery occur approx. once for every 10^7 nucleotides during replication [99] and DNA oxidation through ROS takes place (endogenous damage). But damage to the genetic material also results from exogenous influences such as ionizing radiation (IR), UV, toxins, mutagenic chemicals and there are several repair pathways for the various kinds of mistakes that can occur. Non-homologous end-joining (NHEJ) is responsible for the repair of the majority of double strand breaks (DSBs) in metazoans [90]. It exists with six core factors: Ku (composed of Ku70 and Ku86, a.k.a. Ku80), DNA-PKcs, Artemis, XRCC4, XLF and LIG4 [49]. This DSB repair mechanism operates through microhomologies exposed on single strand overhangs on the DSB ends and results in nucleotide loss due to DNA end trimming by the endonuclease Artemis-DNA-PKcs complex. In contrast to this error-prone repair mechanism, homologous recombination (HR) relies on the completion of resected broken DSB ends by DNA synthesis through DNA polymerase where the intact homologous chromosome serves as a template. This mechanism, however, is only applicable to diploid cells and usually only happens during S phase and G2 phase of the cell division cycle during which the DNA content is $4N$ and sister chromatids are adjacent to each other. Additionally, genomes with a high amount of repetitive sequences (e.g. microsatellites) preferentially use the NHEJ mechanism instead of HR during DNA repair [90]. The damage of single altered nucleotides undergoes different repair

pathways, such as the base excision repair (BER), mismatch repair (MMR), and nucleotide excision repair (NER) pathway. Oxidized nucleotides, for instance, when derived from damage through ROS, were found to be very distinct between the two investigated populations (DNA/RNA oxidation measurements) and naturally undergo the BER mechanism. The core proteins of this pathway are a specific DNA glycosylase, AP endonuclease, end processing enzymes (e.g. PNKP), DNA polymerases, flap endonuclease (FEN), and DNA LIG3 with its cofactor XRCC1. The selected genes that were investigated via qRT-PCR (Tab 4.2) cover some of the core proteins important in oxidative damage related repair pathways.

Table 4.2: DNA repair genes that were investigated via qRT-PCR. Genes coding for core proteins important in oxidative damage response are marked with #.

Gene	Protein name	DNA repair pathway
FEN1 #	flap endonuclease 1	Non-homologous end-joining, Base excision repair
MSH6	mutS homologue 6	Mismatch repair
NBS1 #	nijmegen-breakage-syndrome 1, nib-rin	Homologous recombination, Non-homologous end-joining
XRCC1 #	x-ray repair cross-complementing protein 1	Non-homologous end-joining, Base excision repair
XRCC4 #	x-ray repair cross-complementing protein 4	Non-homologous end-joining
NHEJ1 #	Non-homologous end-joining 1, a.k.a. XLF	Non-homologous end-joining
EXO1	exonuclease 1	Mismatch repair, Homologous recombination

FEN1 being a member in the NHEJ and BER pathways; it is present in proliferative tissues, at very low levels in quiescent cells, but upregulated in many cancer cells [199] making it indicative for malignant or transformed cells. The **MSH2-MSH6** complex plays a fundamental role in the repair of mismatched DNA bases. It is associated with Ku70 and enhanced by several DSB-inducing agents (neocarzinostatin, IR) [150]. The authors also postulate that **MSH6** expression stimulates and regulates NHEJ. Together with Mre11 and Rad50, **NBS1** is part of the MRN complex which recognizes DSBs and recruits further proteins to the site of lesion making it essential for both DSB repair mechanisms (HR and NHEJ) [77]. Lim *et al.* [91] described an S-phase arrest dependent on the phosphorylation of p95/Nbs1 when in response to IR exposure. **XRCC1** is a core protein of the BER repair pathway and also plays a role in the Ku/LIG4-independent pathway of the NHEJ mechanism. It has been shown that **XRCC1** mRNA and protein levels were increased after DNA damage [194]. Wang *et al.* [182] found that JWA, a novel BER protein in oxidative-stress-induced DNA SSBs, is responsible for the regulation of **XRCC1** at transcriptional and post-transcriptional level. **XRCC4** is known to have an essential role in NHEJ.

It stabilizes DNA LIG4 and may serve to localize DNA LIG4 to the site of DSB repair. Even though a regulation of XRCC4 through other protein modifications (e.g. SUMO modification) has been postulated [195], a slight upregulation of XRCC4 after γ -irradiation has been observed [186] and XRCC4 was still selected as a gene of interest. The NHEJ corefactor **XLF** (NHEJ1) is structurally similar to XRCC4 and stimulates the ligation process of the LIG4/XRCC4 complex. XLF deficient mutants are highly sensitive to IR and mutated cells show increased chromosomal instability following mild replication stress conditions [149]. XLF is crucial for the maintenance of fragile site stability under conditions that perturb DNA replication, but not under normal growth conditions [149]. **EXO1** is a member of the Rad2 family of structure specific nucleases that possess 5'→3' exonuclease and 5' flap endonuclease activity. Biochemical and genetic evidence suggested that EXO1 functions catalytically during MMR, and defects in EXO1 cause genomic instability leading to the development of cancer [171]. The regulation of EXO1 to stresses remains unknown so far, but a moderate effect on longevity has been described [113], making this gene relevant for my investigations presented here.

4.4.2 Gene expression

The detailed gene expression analyses over age in both populations of selected genes of interest discussed above revealed very variable gene expression along investigated ages (Appendix p3 for graphs). Hence, expression profiles, as the observed plateau-up profile (5) showing distinctly differently gene expression over age for Cu/ZnSOD in gill tissue of Iceland transcripts, could not be confirmed. The stable gene expression over age might indicate that ageing per se cannot be regarded as a “stress treatment” for these animals. The constant expression of ageing-associated genes during the complete lifespan of *A. islandica* might possibly be a relevant factor for reaching a long lifespan. Differences of mean gene expression levels over all investigated ages between the two populations could be observed for several investigated genes of interest. Most of these genes were significantly higher expressed in the long-lived Iceland population, such as the DNA repair genes NHEJ1 (a.k.a. XLF) and FEN1, and the longevity-associated genes SIRT1 and p53. There are different types of SODs in eukaryotes of which Cu/ZnSOD is located in the cytosol and in extracellular space, and MnSOD is located in the mitochondria – the major site of ROS generation [55]. Interestingly, the levels of the antioxidant Cu/ZnSOD were significantly stronger ($p < 0.0001$) expressed in the Iceland population, whereas MnSOD was significantly higher ($p \leq 0.05$) expressed in the Baltic Sea population indicating different demands of radical scavengers at different cellular locations in the two populations. The

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DNA repair and ageing-associated gene EXO1 was expressed strongly in the Baltic Sea sampled population. The overall results do not reveal a better DNA repair mechanism or higher antioxidant capacity or demand in either of the two populations. However, it does give a hint towards the different life strategies resulting in different gene expression behavior of the two populations as previously shown by Philipp *et al.* [130]. The authors detected distinct expression behavior of Baltic Sea *A. islandica* during the reoxygenation process after MRD compared to a longer-lived *A. islandica* population from the North Sea. A deeper look into investigated genes and pathways need to be performed for a better understanding on each population's survival strategy. But due to annotation restrictions this approach is admittedly limited to very few genes, and key components e.g. Mre11, Ku70, DNA LIG4 and LIG1 may not be identifiable.

5 Conclusion and Outlook

This study of comparing two *A. islandica* populations with vast differences in MLSPs has revealed that the two populations do not substantially differ regarding oxidative damage, molecular ageing markers or expression behaviour of selected genes over age. Significantly higher levels of lipid peroxidation in the long-lived Iceland population might be ascribed to the colder environment and therefore potentially different lipid composition. This finding strongly requires further investigation, such as changes in lipid composition under experimental and natural exposure to different temperatures, to draw further conclusions. One parameter, however, might have been revealed which may cause the vast difference in MLSP. Most strikingly, oxidation of RNA and DNA accumulated much faster, and levels of DNA damage were significantly higher in the Baltic Sea population when compared to the Iceland population. The recurring events of hypoxia/anoxia in the warmer Baltic Sea environment might cause these remarkable differences. In contrast to a longer-lived German Bight population which suppressed transcription in hypoxia/anoxia-related genes during oxygen deficiency, the Baltic Sea population increased transcription – an energy consuming process [130]. Remarkably, the presented study showed significantly higher gene expression levels of MnSOD that operates in mitochondria, where most ROS are produced through respiration, in the Baltic Sea as compared to the Iceland population. In contrast, Cu/ZnSOD found in the cytosol, nucleus, and peroxisomes was significantly higher expressed in the long-lived Iceland population. These results corroborate findings by Orr & Sohal [119] and Bayne *et al.* [17]. Orr & Sohal [119] extended *Drosophila* lifespan by overexpression of Cu/ZnSOD and CAT, whereas Bayne *et al.* [17] could not increase *Drosophila* lifespan when they overexpressed MnSOD and CAT. The higher expression of mitochondrial SOD in the Baltic Sea population observed in this study could be explained by a greater demand of antioxidant due to a higher level of ROS as Baltic Sea individuals do not seem to down-regulate their metabolism during hypoxic/anoxic events. In plants, fungi and protists an alternative oxidase (AOX) for the reduction of oxygen in the electron transport chain exists and has been identified in several animal phyla amongst which are also Mollusca [100]. In addition to cytochrome c oxidase, AOX has been identified in the combined *A. islandica* transcriptome and was represented with >60 % of its reads

by Iceland individuals indicating a higher expression in the long-lived population. A detailed gene expression analysis via qRT-PCR could reveal information on whether both populations show distinct respiration mechanisms.

The higher expression of Cu/ZnSOD in the Iceland population might explain the significantly lower level of DNA damage that occurs in the nucleus. It is also known that mitochondrial DNA (mtDNA) is much more oxidized with age than nuclear DNA (nDNA) [135] which can be further confirmed by measuring nDNA and mtDNA oxidation levels in these two populations. Interestingly, genes significantly upregulated in the Iceland compared to Baltic Sea population either play a role in ROS-related DNA repair pathways (e.g. NHEJ, FEN1), ROS scavenging in the nucleus (Cu/ZnSOD), ROS and DNA damage response (e.g. p53), or in the transcriptional regulation (e.g. SIRT1 by the modification of histones) (Tab 5.1). The roles of these genes in DNA repair mechanisms are in agreement with Nebel *et al.* [113] and Karanjawala & Lieber [90] who stated that many ageing syndromes are caused by a failure in the DNA repair machinery that makes this process very relevant for ageing research. However, a close relationship between the organism's environment (e.g. temperature, oxygen supply) and its gene response is also strongly indicated.

Table 5.1: Significantly differential expressed genes in the two investigated *A. islandica* populations. Levels of significances are indicated as * = $p \leq 0.05$, ** = $p < 0.01$, *** = $p < 0.0001$. n.a. = not applicable.

Category	Upregulated in Iceland	Relevant pathway/process involved	Upregulated in Baltic Sea	Relevant pathway/process involved
DNA repair	NHEJ1 ** (XLF) FEN1 *	NHEJ NHEJ, BER	EXO1 *	MMR, HR
Ageing-relevant	SIRT1 ** p53 *	cancer, stress response, metabolism, deacetylation of histones cell cycle regulation, apoptosis, ROS and DNA damage response	n.a.	n.a.
Antioxidant	Cu/Zn SOD ***	ROS scavenging in: cytosol, nucleus, peroxisomes	MnSOD *	ROS scavenging in: mitochondria

Mechanisms of the ageing-associated genes p53 and SIRT1 are not well understood so far, but an interplay of both has been proposed many times [50, 116, 89]. Thus, the upregulation of both genes in the Iceland population implies a life-extending effect, even though whether up- or down-regulation of p53 prolongs lifespan remains

controversial [50]. Both ageing-associated genes interact with and regulate forkhead box O3 or FOXO3 [50, 112], a known longevity-associated gene [52, 8] involved in the protection from oxidative stress through the upregulation of antioxidants, such as MnSOD and catalase [50]. The FOXO gene could also be identified in the combined *A. islandica* transcriptome database, where almost $\frac{2}{3}$ of the reads originate from Iceland and $\frac{1}{3}$ from the Baltic Sea, indicating an over-expression in the long-lived population. These findings, however, must be confirmed via qRT-PCR since gene expression derived from the transcriptome was found to be rather unreliable. EXO1 was found to be a moderate longevity-associated gene [113] responsible for genomic stability and DNA damage repair through MMR and HR. This effect was ascribed to a certain gene variant predominantly observed in centenarians and not due to gene expression intensities. One can speculate that an overexpression of this genome-maintenance gene in the Baltic Sea population might hence be attributed to the efforts of Baltic Sea individuals to preserve their genome from damaging environmental influences. The remarkable difference in DNA and RNA oxidation between the two investigated populations also indicates genomic impairment due to extrinsic influences. Interestingly, nucleotide oxidation accumulated at the same pace in both populations when considering their respective relative lifespans (Fig 3.10).

The oxidative damage to nucleotides, the effort of damage avoidance through ROS scavengers (Cu/ZnSOD and MnSOD), the consequent struggle of DNA damage repair through NHEJ and BER, and the significant differences in regulatory genes (p53, SIRT1), together with the observation of similar damage accumulation regarding relative lifespan of both populations point towards a decisive interaction between environmental factors causing extreme differences in MSLP within the same species. Further study in the ageing of a Baltic Sea individual in the Iceland environment or vice versa would be an eye-opening experiment. However, the duration of 40 or 400 years for the outcome of such a study is not physically feasible. Nevertheless, whether epigenetic factors might be established in populations over generations is an extraordinarily valuable question. Thus, the investigation of epigenetically heritable longevity mechanisms in model organisms with shorter generation times would be very elucidative. However, epigenetic changes to the genome can at the most be implied but were not studied in this project. The reliable statements obtained from this study is a distinct gene expression behaviour between the long-lived Iceland and the short-lived Baltic Sea *A. islandica* populations and a much higher susceptibility to oxidative damage of nucleotides in the shorter-lived Baltic Sea population. These results highly suggest further research in the differential gene expression behaviour of the two unlike mollusk populations regarding identified DNA repair and antioxidant coding genes as a next step leading to a more healthy ageing.

Bibliography

- [1] D. Abele, T. Brey, and E. Philipp. Bivalve models of aging and the determination of molluscan lifespans. *Experimental gerontology*, 44(5):307–315, 2009.
- [2] D. Abele, J. Strahl, T. Brey, and E. E. Philipp. Imperceptible senescence: ageing in the ocean quahog *Arctica islandica*. *Free radical research*, 42(5):474–480, 2008.
- [3] J. M. Adams and M. White. Biological ageing a fundamental, biological link between socio-economic status and health? *The European Journal of Public Health*, 14(3):331–334, 2004.
- [4] S. S. Agarwal, D. K. Dube, and L. A. Loeb. On the fidelity of DNA replication. Accuracy of *Escherichia coli* DNA polymerase. *Journal of Biological Chemistry*, 254(1):101–106, 1979.
- [5] C. L. Andersen, J. L. Jensen, and T. F. Ørntoft. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research*, 64(15):5245–5250, 2004.
- [6] B. Andziak and R. Buffenstein. Disparate patterns of age-related changes in lipid peroxidation in long-lived naked mole-rats and shorter-lived mice. *Aging cell*, 5(6):525–532, 2006.
- [7] B. Andziak, T. P. O’Connor, W. Qi, E. M. DeWaal, A. Pierce, A. R. Chaudhuri, H. Van Remmen, and R. Buffenstein. High oxidative damage levels in the longest-living rodent, the naked mole-rat. *Aging cell*, 5(6):463–471, 2006.
- [8] C. V. Anselmi, A. Malovini, R. Roncarati, V. Novelli, F. Villa, G. Condorelli, R. Bellazzi, and A. A. Puca. Association of the FOXO3A locus with extreme longevity in a southern Italian centenarian study. *Rejuvenation research*, 12(2):95–104, 2009.
- [9] O. S. Astthorsson, A. Gislason, and S. Jonsson. Climate variability and the Icelandic marine ecosystem. *Deep Sea Research Part II: Topical Studies in Oceanography*, 54(23):2456–2477, 2007.
- [10] G. Aubert and P. M. Lansdorp. Telomeres and aging. *Physiological reviews*, 88(2):557–579, 2008.
- [11] S. Austad. Methusaleh’s Zoo: how nature provides us with clues for extending human health span. *Journal of Comparative Pathology*, 142:S10–S21, 2010.
- [12] S. N. Austad. Is there a role for new invertebrate models for aging research? *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 64(2):192, 2009.
- [13] S. N. Austad. Cats, ‘rats’, and bats: The comparative biology of aging in the 21st century. *Integrative and Comparative Biology*, 50(5):783–792, 2010.
- [14] B. A. C. C. Author and Team. *Assessment of climate change for the Baltic Sea Basin*, volume 2. Springer, Berlin Heidelberg, 2011.
- [15] G. T. Baker and R. L. Sprott. Biomarkers of aging. *Experimental gerontology*, 23(4):223–239, 1988.

Bibliography

- [16] L. Basova, S. Begum, J. Strahl, A. Sukhotin, T. Brey, E. Philipp, and D. Abele. Age-dependent patterns of antioxidants in *Arctica islandica* from six regionally separate populations with different lifespans. *Aquatic Biology*, 14:141–152, 2012.
- [17] A. C. V. Bayne, R. J. Mockett, W. C. Orr, and R. S. Sohal. Enhanced catabolism of mitochondrial superoxide/hydrogen peroxide and aging in transgenic *Drosophila*. *Biochemical Journal*, 391(Pt 2):277, 2005.
- [18] S. Begum, L. Basova, O. Heilmayer, E. E. Philipp, D. Abele, and T. Brey. Growth and energy budget models of the bivalve *Arctica islandica* at six different sites in the Northeast Atlantic realm. *Journal of Shellfish Research*, 29(1):107–115, 2010.
- [19] S. Begum, L. Basova, J. Strahl, A. Sukhotin, O. Heilmayer, E. Philipp, T. Brey, and D. Abele. A metabolic model for the ocean quahog *Arctica islandica* - effects of animal mass and age, temperature, salinity, and geography on respiration rate. *Journal of Shellfish Research*, 28(3):533–539, 2009.
- [20] C. D. Belair, T. R. Yeager, P. M. Lopez, and C. A. Reznikoff. Telomerase activity: a biomarker of cell proliferation, not malignant transformation. *Proceedings of the National Academy of Sciences*, 94(25):13677–13682, 1997.
- [21] Y. Benjamini and Y. Hochberg. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, pages 289–300, 1995.
- [22] B. S. Berlett and E. R. Stadtman. Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry*, 272(33):20313–20316, 1997.
- [23] V. A. Bohr. Repair of oxidative dna damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. *Free radical biology & medicine*, 32(9):804, 2002.
- [24] J. A. Bolker. Model systems in developmental biology. *BioEssays*, 17(5):451–455, 1995.
- [25] V. Bollati, A. Baccarelli, L. Hou, M. Bonzini, S. Fustinoni, D. Cavallo, H.-M. Byun, J. Jiang, B. Marinelli, A. C. Pesatori, et al. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer research*, 67(3):876–880, 2007.
- [26] F. M. Bollmann. The many faces of telomerase: emerging extratelomeric effects. *Bioessays*, 30(8):728–732, 2008.
- [27] R. L. Bowen and C. S. Atwood. Living and dying for sex. *Gerontology*, 50(5):265–290, 2004.
- [28] T. Brey, W. Arntz, D. Pauly, and H. Rumohr. *Arctica (Cyprina) islandica* in Kiel Bay (Western Baltic): growth, production and ecological significance. *Journal of experimental marine biology and ecology*, 136(3):217–235, 1990.
- [29] T. Brown. *Genomes*. Wiley-Liss, Oxford, 2002. 2nd edition.
- [30] K. J. Buchkovich and C. W. Greider. Telomerase regulation during entry into the cell cycle in normal human T cells. *Molecular biology of the cell*, 7(9):1443, 1996.
- [31] R. Buffenstein. Negligible senescence in the longest living rodent, the naked mole-rat: insights from a successfully aging species. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, 178(4):439–445, 2008.
- [32] R. N. Butler, R. Sprott, H. Warner, J. Bland, R. Feuers, M. Forster, H. Fillit, S. M. Harman, M. Hewitt, and M. Hyman. Aging: The Reality Biomarkers of Aging: From Primitive Organisms to Humans. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 59(6):B560–B567, 2004.

Bibliography

- [33] F. Cabreiro, D. Ackerman, R. Doonan, C. Araiz, P. Back, D. Papp, B. P. Braeckman, and D. Gems. Increased life span from overexpression of superoxide dismutase in *Caenorhabditis elegans* is not caused by decreased oxidative damage. *Free Radical Biology and Medicine*, 51(8):1575–1582, 2011.
- [34] K. W. Caldecott. Mammalian single-strand break repair: mechanisms and links with chromatin. *DNA repair*, 6(4):443–453, 2007.
- [35] J. Campisi, S.-h. Kim, C.-S. Lim, and M. Rubio. Cellular senescence, cancer and aging: the telomere connection. *Experimental gerontology*, 36(10):1619–1638, 2001.
- [36] A. R. Chaudhuri, E. M. de Waal, A. Pierce, H. Van Remmen, W. F. Ward, and A. Richardson. Detection of protein carbonyls in aging liver tissue: A fluorescence-based proteomic approach. *Mechanisms of ageing and development*, 127(11):849–861, 2006.
- [37] L. Chen, S. Huang, L. Lee, A. Davalos, R. H. Schiestl, J. Campisi, and J. Oshima. WRN, the protein deficient in werner syndrome, plays a critical structural role in optimizing DNA repair. *Ageing cell*, 2(4):191–199, 2003.
- [38] M. Chevion, E. Berenshtein, and E. Stadtman. Human studies related to protein oxidation: Protein carbonyl content as a marker of damage. *Free radical research*, 33:S99, 2000.
- [39] K. Christensen, T. E. Johnson, and J. W. Vaupel. The quest for genetic determinants of human longevity: challenges and insights. *Nature Reviews Genetics*, 7(6):436–448, 2006.
- [40] D. J. Conley, S. Björck, E. Bonsdorff, J. Carstensen, G. Destouni, B. G. Gustafsson, S. Hietaanen, M. Kortekaas, H. Kuosa, H. E. M. Meier, B. Müller-Karulis, K. Nordberg, A. Norkko, G. Nürnberg, H. Pitkänen, N. N. Rabalais, R. Rosenberg, O. P. Savchuk, C. P. Slomp, M. Voss, F. Wulff, and L. Zillen. Critical Review Hypoxia-Related Processes in the Baltic Sea. *Environ. Sci. Technol*, 43(10):3412–3420, 2009.
- [41] M. S. Cooke, R. Rozalski, R. Dove, D. Gackowski, A. Siomek, M. D. Evans, and R. Olinski. Evidence for attenuated cellular 8-oxo-7, 8-dihydro-2'-deoxyguanosine removal in cancer patients. *Biological chemistry*, 387(4):393–400, 2006.
- [42] E. L. Crockett. The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. *Journal of Comparative Physiology B*, 178(7):795–809, 2008.
- [43] T. Dahlgren, J. Weinberg, and K. Halanych. Phylogeography of the ocean quahog (*Arctica islandica*): influences of paleoclimate on genetic diversity and species range. *Marine Biology*, 137(3):487–495, 2000.
- [44] J. De Boer and J. H. Hoeijmakers. Nucleotide excision repair and human syndromes. *Carcinogenesis*, 21(3):453–460, 2000.
- [45] J. P. de Magalhães, C. E. Finch, and G. Janssens. Next-generation sequencing in aging research: emerging applications, problems, pitfalls and possible solutions. *Ageing research reviews*, 9(3):315, 2010.
- [46] R. Development and C. o. r. e. Team. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, 2008.
- [47] J. W. Dippner, C. Möller, and J. Hänninen. Regime shifts in North Sea and Baltic Sea: A comparison. *Journal of Marine Systems*, 2012.
- [48] S. L. Estabrooks. The possible role of telomeres in the short life span of the bay scallop, *Argopecten irradians irradians* (Lamarck 1819). *Journal of Shellfish Research*, 26(2):307–313, 2007.

Bibliography

- [49] F. J. Fattah. *The impact of loss of function mutations of NHEJ genes on gene targeting and DNA DSB repair in human somatic cells*. Dissertation, University of Minnesota, 2009.
- [50] Z. Feng, M. Lin, and R. Wu. The Regulation of Aging and Longevity A New and Complex Role of p53. *Genes & Cancer*, 2(4):443–452, 2011.
- [51] C. Finch and S. N. Austad. History and prospects: symposium on organisms with slow aging. *Experimental gerontology*, 36(4):593–597, 2001.
- [52] F. Flachsbart, A. Caliebe, R. Kleindorp, H. Blanché, H. von Eller-Eberstein, S. Nikolaus, S. Schreiber, and A. Nebel. Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proceedings of the National Academy of Sciences*, 106(8):2700–2705, 2009.
- [53] N. R. Forsyth, W. E. Wright, and J. W. Shay. Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again. *Differentiation*, 69(4-5):188–197, 2002.
- [54] N. Francis, T. Gregg, R. Owen, T. Ebert, and A. Bodnar. Lack of age-associated telomere shortening in long- and short-lived species of sea urchins. *FEBS letters*, 580(19):4713–4717, 2006.
- [55] I. Fridovich. Superoxide radical and superoxide dismutases. *Annual review of biochemistry*, 64(1):97–112, 1995.
- [56] L. Y. Geer, M. Domrachev, D. J. Lipman, and S. H. Bryant. CDART: protein homology by domain architecture. *Genome research*, 12(10):1619–1623, 2002.
- [57] A. P. Grollman and M. Moriya. Mutagenesis by 8-oxoguanine: an enemy within. *Trends in Genetics*, 9(7):246–249, 1993.
- [58] L. Guarente. Sirtuins, aging, and medicine. *New England Journal of Medicine*, 364(23):2235–2244, 2011.
- [59] L. Guarente and C. Kenyon. Genetic pathways that regulate ageing in model organisms. *Nature*, pages 255–262, 2000.
- [60] X. Guo and S. K. Allen Jr. Fluorescence in situ hybridization of vertebrate telomere sequence to chromosome ends of the Pacific oyster, *Crassostrea gigas* Thunberg. *Journal of Shellfish Research*, 16(1):87–90, 1997.
- [61] S. R. Haley. Sex ratio as a function of size in *Hippa pacifica* Dana (Crustacea, Anomura, Hippidae): a test of the sex reversal and differential growth rate hypotheses. *American naturalist*, pages 391–397, 1979.
- [62] M. L. Hamilton, H. Van Remmen, J. A. Drake, H. Yang, Z. M. Guo, K. Kewitt, C. A. Walter, and A. Richardson. Does oxidative damage to DNA increase with age? *Proceedings of the National Academy of Sciences*, 98(18):10469–10474, 2001.
- [63] S. D. Han and M. W. Bondi. Revision of the apolipoprotein E compensatory mechanism recruitment hypothesis. *Alzheimer's & Dementia*, 4(4):251–254, 2008.
- [64] C. B. Harley, A. B. Futcher, and C. W. Greider. Telomeres shorten during ageing of human fibroblasts. *Nature*, 345:458–460, 1990.
- [65] D. Harman. *Aging: a theory based on free radical and radiation chemistry*. University of California Radiation Laboratory, 1955.
- [66] L. Hayflick. The limited *in vitro* lifetime of human diploid cell strains. *Experimental cell research*, 37:614–636, 1965.

Bibliography

- [67] T. Helleday, J. Lo, D. C. van Gent, and B. P. Engelward. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA repair*, 6(7):923–935, 2007.
- [68] J. H. Hoeijmakers. DNA damage, aging, and cancer. *New England Journal of Medicine*, 361(15):1475–1485, 2009.
- [69] T. Hofer, A. Y. Seo, M. Prudencio, and C. Leeuwenburgh. A method to determine RNA and DNA oxidation simultaneously by HPLC-ECD: greater RNA than DNA oxidation in rat liver after doxorubicin administration. *Biological chemistry*, 387(1):103–111, 2006.
- [70] S. P. Holmes, R. Witbaard, and J. van der Meer. Phenotypic and genotypic population differentiation in the bivalve mollusc *Arctica islandica*: results from RAPD analysis. *Marine Ecology Progress Series*, 254:163–176, 2003.
- [71] N. R. Hooyman and H. A. Kiyak. *Social gerontology: A multidisciplinary perspective*. Pearson/Allyn & Bacon, Boston, 2008.
- [72] D. M. Huffman, D. R. Moellering, W. E. Grizzle, C. R. Stockard, M. S. Johnson, and T. R. Nagy. Effect of exercise and calorie restriction on biomarkers of aging in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 294(5):R1618–R1627, 2008.
- [73] A. K. Hüning, F. Melzner, J. Thomsen, M. A. Gutowska, L. Krämer, S. Frickenhaus, P. Rosenstiel, H.-O. Pörtner, E. E. Philipp, and M. Lucassen. Impacts of seawater acidification on mantle gene expression patterns of the Baltic Sea blue mussel: implications for shell formation and energy metabolism. *Marine Biology*, pages 1–17, 2012.
- [74] G. Husmann, D. Abele, P. Rosenstiel, M. Clark, L. Kraemer, and E. Philipp. Age dependent expression of stress and antimicrobial genes in the hemocytes and siphon tissue of the Antarctic bivalve, *Laternula elliptica*, exposed to injury and starvation. *Cell Stress & Chaperones*, in revision.
- [75] C. Jiang and J.-Y. Chang. Isomers of human α -synuclein stabilized by disulfide bonds exhibit distinct structural and aggregative properties. *Biochemistry*, 46(2):602–609, 2007.
- [76] C. Jüngst, B. Cheng, R. Gehrke, V. Schmitz, H. D. Nischalke, J. Ramakers, P. Schramel, P. Schirmacher, T. Sauerbruch, and W. H. Caselmann. Oxidative damage is increased in human liver tissue adjacent to hepatocellular carcinoma. *Hepatology*, 39(6):1663–1672, 2004.
- [77] K. A. Karen, P. J. Hoey, C. Young, and P. Hearing. Temporal regulation of the Mre11-Rad50-Nbs1 complex during adenovirus infection. *Journal of virology*, 83(9):4565–4573, 2009.
- [78] M. Kasapoglu, T. Ozben, et al. Alterations of antioxidant enzymes and oxidative stress markers in aging. *Experimental gerontology*, 36(2):209, 2001.
- [79] N. W. Kim, M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. d. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, and J. W. Shay. Specific association of human telomerase activity with immortal cells and cancer. *Science (New York, NY)*, 266(5193):2011, 1994.
- [80] T. B. Kirkwood. Evolution of ageing. *Nature*, 270(5635):301, 1977.
- [81] W. Klapper, K. Heidorn, K. Kühne, R. Parwaresch, and G. Krupp. Telomerase activity in immortal fish. *FEBS letters*, 434(3):409–412, 1998.
- [82] W. Klapper, K. Kühne, K. K. Singh, K. Heidorn, R. Parwaresch, and G. Krupp. Longevity of lobsters is linked to ubiquitous telomerase expression. *FEBS letters*, 439(1):143–146, 1998.

Bibliography

- [83] W. Klapper, R. Parwaresch, and G. Krupp. Telomere biology in human aging and aging syndromes. *Mechanisms of ageing and development*, 122(7):695–712, 2001.
- [84] R. Kolodner. Biochemistry and genetics of eukaryotic mismatch repair. *Genes & development*, 10(12):1433–1442, 1996.
- [85] M. Kuningas, S. P. Mooijaart, D. Van Heemst, B. J. Zwaan, P. E. Slagboom, and R. G. Westendorp. Genes encoding longevity: from model organisms to humans. *Aging Cell*, 7(2):270–280, 2008.
- [86] T. A. Kunkel and D. A. Erie. DNA mismatch repair. *Annu. Rev. Biochem.*, 74:681–710, 2005.
- [87] E. S. Lander, L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, et al. Initial sequencing and analysis of the human genome. *Nature*, 409(6822):860–921, 2001.
- [88] M. Z. Levy, R. C. Allsopp, A. B. Futcher, C. W. Greider, and C. B. Harley. Telomere end-replication problem and cell aging. *Journal of molecular biology*, 225(4):951–960, 1992.
- [89] L. Li, L. Wang, L. Li, Z. Wang, Y. Ho, T. McDonald, T. L. Holyoake, W. Chen, and R. Bhatia. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. *Cancer Cell*, 21(2):266–281, 2012.
- [90] M. R. Lieber and Z. E. Karanjawala. Ageing, repetitive genomes and DNA damage. *Nature Reviews Molecular Cell Biology*, 5(1):69–75, 2004.
- [91] D.-S. Lim, S.-T. Kim, B. Xu, R. S. Maser, J. Lin, J. H. Petrini, and M. B. Kastan. ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature*, 404(6778):613–617, 2000.
- [92] T. Lindahl. Instability and decay of the primary structure of DNA. *Nature*, 362(6422):709–715, 1993.
- [93] J. Loram and A. Bodnar. Age-related changes in gene expression in tissues of the sea urchin *Strongylocentrotus purpuratus*. *Mechanisms of ageing and development*, 2012.
- [94] J. Lü, L. Shi, Z. Wu, Y. Liao, C. Zhou, Y. Li, X. Bin, B. Zeng, and J. Chen. Study of 8-oh-dg and its correlation with several cancer related gene in lung cancer tissues. *Wei sheng yan jiu = Journal of hygiene research*, 32(4):304, 2003.
- [95] R. Mann. The seasonal cycle of gonadal development in *Arctica islandica* from the southern New England shelf. *Fish. Bull*, 80(2):315–326, 1982.
- [96] E. R. Mardis. The impact of next-generation sequencing technology on genetics. *Trends in genetics*, 24(3):133, 2008.
- [97] P. J. Mason. Stem cells, telomerase and dyskeratosis congenita. *Bioessays*, 25(2):126–133, 2003.
- [98] S. Maynard, S. H. Schurman, C. Harboe, N. C. de Souza-Pinto, and V. A. Bohr. Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis*, 30(1):2–10, 2009.
- [99] S. D. McCulloch and T. A. Kunkel. The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell research*, 18(1):148–161, 2008.
- [100] A. E. McDonald and G. C. Vanlerberghe. Branched mitochondrial electron transport in the animalia: presence of alternative oxidase in several animal phyla. *IUBMB life*, 56(6):333–341, 2008.

Bibliography

- [101] Z. A. Medvedev. An attempt at a rational classification of theories of ageing. *Biological Reviews*, 65(3):375–398, 1990.
- [102] A. Merrill and J. Ropes. The general distribution of the surf clam and ocean quahog. In *Proceedings of the National Shellfisheries Association*, volume 59, pages 0–5, 1969.
- [103] J. Meyne, R. L. Ratliff, and R. K. Moyzis. Conservation of the human telomere sequence (TTAGGG)_n among vertebrates. *Proceedings of the National Academy of Sciences*, 86(18):7049–7053, 1989.
- [104] P. M. Mikkelsen, R. Bieler, I. Kappner, and T. A. Rawlings. Phylogeny of Veneroidea (Mollusca: Bivalvia) based on morphology and molecules. *Zoological Journal of the Linnean Society*, 148(3):439–521, 2006.
- [105] J. Miquel, J. Fleming, A. Economos, and J. Johnson. Mitochondrial role in cell aging. *Experimental gerontology*, 15:575–591, 1980.
- [106] P. Montuschi, P. Barnes, and L. Jackson Roberts. Insights into oxidative stress: the isoprostanes. *Current medicinal chemistry*, 14(6):703–717, 2007.
- [107] J. D. Morrow and L. J. Roberts. Mass spectrometric quantification of F₂-isoprostanes in biological fluids and tissues as measure of oxidant stress. *Methods in enzymology*, 300:3–12, 1999.
- [108] A. Mortazavi, B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature methods*, 5(7):621–628, 2008.
- [109] D. Munro and P. U. Blier. The extreme longevity of *Arctica islandica* is associated with increased peroxidation resistance in mitochondrial membranes. *Aging Cell*, 2012.
- [110] S. A. Murawski, J. W. Ropes, and F. M. Serchuk. Growth of the ocean quahog, *Arctica islandica*, in the Middle Atlantic Bight. *Fishery Bulletin*, 80(1):21–34, 1982.
- [111] E. W. Myers, G. G. Sutton, A. L. Delcher, I. M. Dew, D. P. Fasulo, M. J. Flanigan, S. A. Kravitz, C. M. Mobarry, K. H. Reinert, and K. A. Remington. A whole-genome assembly of *Drosophila*. *Science*, 287(5461):2196–2204, 2000.
- [112] T. Nakagawa and L. Guarente. Sirtuins at a glance. *Journal of Cell Science*, 124(6):833–838, 2011.
- [113] A. Nebel, F. Flachsbart, A. Till, A. Caliebe, H. Blanché, A. Arlt, R. Häslér, G. Jacobs, R. Kleindorp, and A. Franke. A functional *EXO1* promoter variant is associated with prolonged life expectancy in centenarians. *Mechanisms of ageing and development*, 130(10):691–699, 2009.
- [114] A. Nebel, R. Kleindorp, A. Caliebe, M. Nothnagel, H. Blanché, O. Junge, M. Wittig, D. Ellinghaus, F. Flachsbart, and H.-E. Wichmann. A genome-wide association study confirms *APOE* as the major gene influencing survival in long-lived individuals. *Mechanisms of ageing and development*, 132(6):324–330, 2011.
- [115] W. L. Neeley and J. M. Essigmann. Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. *Chemical research in toxicology*, 19(4):491, 2006.
- [116] S. Nemoto, M. M. Fergusson, and T. Finkel. Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. *Science Signalling*, 306(5704):2105, 2004.
- [117] D. Nicol. Recent species of the veneroid pelecypod *Arctica*. *Jour. Washington Acad. Sci.*, 41(3):102–106, 1951.

Bibliography

- [118] N. t. l. Oceanic Atmospheric Administration, N. t. l. Marine Fisheries Service, and N. E. Fisheries Science Center. Ocean quahog, *Arctica islandica*, life history and habitat characteristics. NOAA technical memorandum, U. S. Department of Commerce, 1999.
- [119] W. C. Orr and R. S. Sohal. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science (New York, NY)*, 263(5150):1128, 1994.
- [120] M. M. Ouellette, M. Liao, B.-S. Herbert, M. Johnson, S. E. Holt, H. S. Liss, J. W. Shay, and W. E. Wright. Subsenescent telomere lengths in fibroblasts immortalized by limiting amounts of telomerase. *Journal of Biological Chemistry*, 275(14):10072–10076, 2000.
- [121] R. Owen, S. Sarkis, and A. Bodnar. Developmental pattern of telomerase expression in the sand scallop, *Euvola ziczac*. *Invertebrate Biology*, 126(1):40–45, 2007.
- [122] L. Partridge and D. Gems. Benchmarks for ageing studies. *Nature*, 450(7167):165–167, 2007.
- [123] V. I. Pérez, R. Buffenstein, V. Masamsetti, S. Leonard, A. B. Salmon, J. Mele, B. Andziak, T. Yang, Y. Edrey, and B. Friguet. Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. *Proceedings of the National Academy of Sciences*, 106(9):3059–3064, 2009.
- [124] E. Peters, P. Yevich, J. Harshbarger, and G. Zarogian. Comparative histopathology of gonadal neoplasms in marine bivalve molluscs. *Diseases of aquatic organisms*, 20(1):59–76, 1994.
- [125] E. Philipp, T. Brey, O. Heilmayer, D. Abele, and H.-O. Pörtner. Physiological ageing in a temperate and a polar swimming scallop. *Marine Ecology Progress Series*, 307:187–198, 2006.
- [126] E. Philipp, T. Brey, H.-O. Pörtner, and D. Abele. Chronological and physiological ageing in a polar and a temperate mud clam. *Mechanisms of ageing and development*, 126(5):598–609, 2005.
- [127] E. Philipp, L. Kraemer, D. Mountfort, M. Schilhabel, S. Schreiber, and P. Rosenstiel. The Transcriptome Analysis and Comparison Explorer – T-ACE: a platform-independent, graphical tool to process large RNAseq datasets of non-model organisms. *Bioinformatics*, 28(6):777–783, 2012.
- [128] E. E. Philipp and D. Abele. Masters of longevity: lessons from long-lived bivalves—a mini-review. *Gerontology*, 56(1):55–65, 2009.
- [129] E. E. Philipp, L. Kraemer, F. Melzner, A. J. Poustka, S. Thieme, U. Findeisen, S. Schreiber, and P. Rosenstiel. Massively parallel RNA sequencing identifies a complex immune gene repertoire in the lophotrochozoan *Mytilus edulis*. *PLoS one*, 7(3):e33091, 2012.
- [130] E. E. Philipp, W. Wessels, H. Gruber, J. Strahl, A. E. Wagner, I. M. Ernst, G. Rimbach, L. Kraemer, S. Schreiber, and D. Abele. Gene Expression and Physiological Changes of Different Populations of the Long-Lived Bivalve *Arctica islandica* under Low Oxygen Conditions. *PLOS ONE*, 7(9):e44621, 2012.
- [131] A. Pierce, E. deWaal, H. van Remmen, A. Richardson, and A. Chaudhuri. A novel approach for screening the proteome for changes in protein conformation. *Biochemistry*, 45(9):3077–3085, 2006.
- [132] M. Plohl, E. Prats, A. Martínez-Lage, A. González-Tizón, J. Méndez, and L. Cornudella. 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(22):19839–19846, 2002.

Bibliography

- [133] E. N. Powell and R. Mann. Evidence of recent recruitment in the ocean quahog *Arctica islandica* in the Mid-Atlantic Bight. *Journal of Shellfish Research*, 24(2):517–530, 2005.
- [134] K. Prise, S. Davies, and B. Michael. Cell killing and DNA damage in Chinese hamster V79 cells treated with hydrogen peroxide. *International journal of radiation biology*, 55(4):583–592, 1989.
- [135] C. Richter, J.-W. Park, and B. N. Ames. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proceedings of the National Academy of Sciences*, 85(17):6465–6467, 1988.
- [136] L. E. Rikans and K. R. Hornbrook. Lipid peroxidation, antioxidant protection and aging. *Biochimica et biophysica acta*, 1362(2-3):116, 1997.
- [137] W. L. Robinette, J. S. Gashwiler, J. B. Low, and D. A. Jones. Differential mortality by sex and age among mule deer. *The Journal of Wildlife Management*, 21(1):1–16, 1957.
- [138] J. W. Ropes. Procedures for preparing acetate peels and evidence validating the annual periodicity of growth lines formed in the shells of ocean quahogs, *Arctica islandica*. *Marine Fisheries Review*, 46(2):27–35, 1984.
- [139] A. Rosenblueth and N. Wiener. The role of models in science. *Philosophy of Science*, 12(4):316–321, 1945.
- [140] S. Rounsley, P. R. Marri, Y. Yu, R. He, N. Sisneros, J. L. Goicoechea, S. J. Lee, A. Angelova, D. Kudrna, and M. Luo. De novo next generation sequencing of plant genomes. *Rice*, 2(1):35–43, 2009.
- [141] S. Rozen and H. Skaletsky. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol*, 132(3):365–386, 2000.
- [142] A. B. Salmon, S. Leonard, V. Masamsetti, A. Pierce, A. J. Podlutzky, N. Podlutzkaya, A. Richardson, S. N. Austad, and A. R. Chaudhuri. The long lifespan of two bat species is correlated with resistance to protein oxidation and enhanced protein homeostasis. *The FASEB Journal*, 23(7):2317–2326, 2009.
- [143] A. Sancar, L. A. Lindsey-Boltz, K. Ünsal-Kaçmaz, and S. Linn. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annual review of biochemistry*, 73(1):39–85, 2004.
- [144] F. Sanger, S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74(12):5463–5467, 1977.
- [145] S. Scheibe and L. L. Carstensen. Emotional aging: Recent findings and future trends. *The Journals of Gerontology Series B: Psychological Sciences and Social Sciences*, 65(2):135, 2010.
- [146] B. R. Schöne, J. Fiebig, M. Pfeiffer, R. Gleß, J. Hickson, A. L. Johnson, W. Dreyer, and W. Oschmann. Climate records from a bivalved Methuselah (*Arctica islandica*, Mollusca; Iceland). *Palaeogeography, Palaeoclimatology, Palaeoecology*, 228(1):130–148, 2005.
- [147] B. R. Schöne, W. Oschmann, J. Rössler, A. D. F. Castro, S. D. Houk, I. Kröncke, W. Dreyer, R. Janssen, H. Rumohr, and E. Dunca. North Atlantic Oscillation dynamics recorded in shells of a long-lived bivalve mollusk. *Geology*, 31(12):1037–1040, 2003.
- [148] J. Schultz, F. Milpetz, P. Bork, and C. P. Ponting. SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences*, 95(11):5857–5864, 1998.

Bibliography

- [149] M. Schwartz, Y. S. Oren, A. C. Bester, A. Rahat, R. Sfez, S. Yitzchaik, J.-P. De Villartay, and B. Kerem. Impaired replication stress response in cells from immunodeficiency patients carrying Cernunnos/XLF mutations. *PLoS one*, 4(2):e4516, 2009.
- [150] A. Shahi, J.-H. Lee, Y. Kang, S. H. Lee, J.-W. Hyun, I.-Y. Chang, J.-Y. Jun, and H. J. You. Mismatch-repair protein MSH6 is associated with Ku70 and regulates DNA double-strand break repair. *Nucleic acids research*, 39(6):2130–2143, 2011.
- [151] J. Shaw, A. Large, P. Donkin, S. Evans, F. Staff, D. Livingstone, J. Chipman, and L. Peters. Seasonal variation in cytochrome P450 immunopositive protein levels, lipid peroxidation and genetic toxicity in digestive gland of the mussel *Mytilus edulis*. *Aquatic toxicology*, 67(4):325–336, 2004.
- [152] L. Shi, D. R. Palleros, and A. L. Fink. Protein conformational changes induced by 1, 1'-bis(4-anilino-5-naphthalenesulfonic acid): preferential binding to the molten globule of DnaK. *Biochemistry*, 33(24):7536–7546, 1994.
- [153] H. N. Sköld, M. E. Asplund, C. A. Wood, and J. D. Bishop. Telomerase deficiency in a colonial ascidian after prolonged asexual propagation. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 316(4):276–283, 2011.
- [154] T. W. Snell, A. M. Fields, and R. K. Johnston. Antioxidants can extend lifespan of *Brachionus manjavacas* (Rotifera), but only in a few combinations. *Biogerontology*, pages 1–15, 2012.
- [155] R. S. Sohal. Role of oxidative stress and protein oxidation in the aging process. *Free Radical Biology and Medicine*, 33(1):37–44, 2002.
- [156] R. S. Sohal, H.-H. Ku, S. Agarwal, M. J. Forster, and H. Lal. Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mechanisms of ageing and development*, 74(1):121–133, 1994.
- [157] E. Southern. Detection of specific sequence among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.*, 98:503–517, 1975.
- [158] S. Steingrímsson and G. Thorarinsdóttir. Age structure, growth and size at sexual maturity in ocean quahog, *Arctica islandica* (Mollusca: Bivalvia), off NW-Iceland. *CM-International Council for the Exploration of the Sea*, 900(K: 54), 1995.
- [159] J. Strahl and D. Abele. Cell turnover in tissues of the long-lived ocean quahog *Arctica islandica* and the short-lived scallop *Aequipecten opercularis*. *Marine Biology*, 157(6):1283–1292, 2010.
- [160] J. Strahl, T. Brey, E. E. Philipp, G. Thorarinsdóttir, N. Fischer, W. Wessels, and D. Abele. Physiological responses to self-induced burrowing and metabolic rate depression in the ocean quahog *Arctica islandica*. *The Journal of Experimental Biology*, 214(24):4223–4233, 2011.
- [161] J. Strahl, E. Philipp, T. Brey, K. Broeg, and D. Abele. Physiological aging in the Icelandic population of the ocean quahog *Arctica islandica*. *Aquatic Biology*, 1(1):77–83, 2007.
- [162] B. L. Strehler. *Time, cells, and aging*. Academic Press, New York, 1963.
- [163] A. Sukhotin, D. Abele, and H.-O. Pörtner. Growth, metabolism and lipid peroxidation in *Mytilus edulis* L.: age and size effects. *Marine ecology-progress series*, 226:223–234, 2002.
- [164] C. Sun, Y. Li, Q. Wu, H. Luo, Y. Sun, J. Song, E. Lui, and S. Chen. De novo sequencing and analysis of the American ginseng root transcriptome using a GS FLX Titanium platform to discover putative genes involved in ginsenoside biosynthesis. *BMC genomics*, 11(1):262, 2010.
- [165] E. Svendsen, A. Aglen, S. Iversen, D. Skagen, and O. Smestad. Influence of climate on

Bibliography

- recruitment and migration of fish stocks in the North Sea. *Can Spec Publ Fish Aquat Sci*, 121:641–653, 1995.
- [166] S. Taber III. The frequency of multiple mating of queen honey bees. *Journal of Economic Entomology*, 47(6):995–998, 1954.
- [167] M. Takata, M. S. Sasaki, E. Sonoda, C. Morrison, M. Hashimoto, H. Utsumi, Y. Yamaguchi-Iwai, A. Shinohara, and S. Takeda. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *The EMBO journal*, 17(18):5497–5508, 1998.
- [168] A. Taylor. Burrowing behaviour and anaerobiosis in the bivalve *Arctica islandica* (L.). *J. Mar. Biol. Assoc. UK*, 56(01):95–109, 1976.
- [169] I. Thompson, D. Jones, and J. Ropes. Advanced age for sexual maturity in the ocean quahog *Arctica islandica* (Mollusca: Bivalvia). *Marine Biology*, 57(1):35–39, 1980.
- [170] G. G. Thorarinsdottir and S. A. Steingrímsson. Size and age at sexual maturity and sex ratio in ocean quahog, *Arctica islandica* (Linnaeus, 1767), off northwest Iceland. *Journal of shellfish research*, 19(2):943–948, 2000.
- [171] P. T. Tran, N. Erdeniz, L. S. Symington, and R. M. Liskay. EXO1 – A multi-tasking eukaryotic nuclease. *DNA repair*, 3(12):1549, 2004.
- [172] W. Traut, M. Szczepanowski, M. Vítková, C. Opitz, F. Marec, and J. Zrzavý. The telomere repeat motif of basal Metazoa. *Chromosome Research*, 15(3):371–382, 2007.
- [173] E. R. Tuminello and S. D. Han. The apolipoprotein e antagonistic pleiotropy hypothesis: review and recommendations. *International journal of Alzheimer’s disease*, 2011, 2011.
- [174] H. Turner. The Mahogany Quahog resource of Massachusetts: Report on investigation of methods of improving the shellfish resources of Massachusetts. *Mass. Dept. of Conservation, Div. of Mar. Fish*, pages 12–16, 1949.
- [175] A. Turturro, W. W. Witt, S. Lewis, B. S. Hass, R. D. Lipman, and R. W. Hart. Growth curves and survival characteristics of the animals used in the Biomarkers of Aging Program. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 54(11):B492–B501, 1999.
- [176] R. Twyman. What are ‘model organisms’? http://genome.wellcome.ac.uk/doc_WTD020803.html, Feb. 2013.
- [177] E. Ungewitter and H. Scoble. Antagonistic pleiotropy and p53. *Mechanisms of ageing and development*, 130(1):10–17, 2009.
- [178] H. Van Remmen, M. L. Hamilton, and A. Richardson. Oxidative damage to DNA and aging. *Exercise and sport sciences reviews*, 31(3):149–153, 2003.
- [179] J. W. Vaupel, J. R. Carey, K. Christensen, T. E. Johnson, A. I. Yashin, N. V. Holm, I. A. Iachine, V. Kannisto, A. A. Khazaeli, P. Liedo, et al. Biodemographic trajectories of longevity. *Science*, 280(5365):855–860, 1998.
- [180] J. Vina, C. Borrás, and J. Miquel. Theories of ageing. *IUBMB life*, 59(4-5):249–254, 2007.
- [181] A. D. J. Wanamaker, J. Heinemeier, J. D. Scourse, C. A. Richardson, P. G. Butler, J. Eiríksson, and K. L. Knudsen. Very long-lived mollusks confirm 17th century AD tephra-based radiocarbon reservoir ages for north Icelandic shelf waters. *Radiocarbon*, 50(3):399–412, 2008.

Bibliography

- [182] S. Wang, Z. Gong, R. Chen, Y. Liu, A. Li, G. Li, and J. Zhou. Jwa regulates XRCC1 and functions as a novel base excision repair protein in oxidative-stress-induced DNA single-strand breaks. *Nucleic acids research*, 37(6):1936–1950, 2009.
- [183] J. Watson. Origin of Concatemeric T7 DNA. *Nature new Biol*, 239, 1972.
- [184] A. M. Wenner. Sex ratio as a function of size in marine Crustacea. *American Naturalist*, pages 321–350, 1972.
- [185] W. Wessels. Investigation of gene expression patterns in *Arctica islandica* (Mollusca, Bivalvia) during phases of metabolic rate depression and hypoxia/anoxia-reoxygenation events. Master’s thesis, University Bremen, Fakultý 2, 2011.
- [186] C. E. West, W. M. Waterworth, Q. Jiang, and C. M. Bray. Arabidopsis DNA ligase IV is induced by γ -irradiation and interacts with an arabidopsis homologue of the double strand break repair protein XRCC4. *The Plant Journal*, 24(1):67–78, 2000.
- [187] K. Wetterstrand. TDNA Sequencing Costs: Data from the NHGRI Genome Sequencing Program (GSP), Feb. 2013.
- [188] D. Wilhelm Filho, S. L. Althoff, A. L. Dafré, and A. Boveris. Antioxidant defenses, longevity and ecophysiology of South American bats. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 146(1):214–220, 2007.
- [189] G. C. Williams. Pleiotropy, natural selection, and the evolution of senescence. *Science’s SAGE KE*, 2001(1):13, 2001.
- [190] D. L. Wingard. The sex differential in mortality rates demographic and behavioral factors. *American Journal of Epidemiology*, 115(2):205–216, 1982.
- [191] R. Witbaard, G. Duineveld, and P. De Wilde. Geographical differences in growth rates of *Arctica islandica* (Mollusca: Bivalvia) from the North Sea and adjacent waters. *Journal of the Marine Biological Association of the UK*, 79(5):907–915, 1999.
- [192] R. Witbaard, M. Jenness, K. Van Der Borg, and G. Ganssen. Verification of annual growth increments in *Arctica islandica* L. from the North Sea by means of oxygen and carbon isotopes. *Netherlands Journal of Sea Research*, 33(1):91–101, 1994.
- [193] M. L. Wolfe. Mortality patterns in the Isle Royale moose population. *American Midland Naturalist*, pages 267–279, 1977.
- [194] A. Yacoub, R. McKinstry, D. Hinman, T. Chung, P. Dent, and M. P. Hagan. Epidermal growth factor and ionizing radiation up-regulate the DNA repair genes XRCC1 and ERCC1 in DU145 and LNCaP prostate carcinoma through MAPK signaling. *Radiation research*, 159(4):439–452, 2003.
- [195] V. Yurchenko, Z. Xue, and M. J. Sadofsky. Sumo modification of human XRCC4 regulates its localization and function in DNA double-strand break repair. *Molecular and cellular biology*, 26(5):1786–1794, 2006.
- [196] I. A. Zalenskaya and A. O. Zalensky. Telomeres in mammalian male germline cells. *International review of cytology*, 218:37–67, 2002.
- [197] E. M. Zdobnov and R. Apweiler. InterProScan—an integration platform for the signature-recognition methods in InterPro. *Bioinformatics*, 17(9):847–848, 2001.
- [198] M. L. Zettler, R. Bonsch, and F. Gosselck. Distribution, abundance, and some population characteristics of the ocean quahog, *Arctica islandica* (Linnaeus, 1767), in the Mecklenburg Bight (Baltic Sea). *Journal of Shellfish Research*, 20(1):161–170, 2001.

Bibliography

- [199] L. Zheng, J. Jia, L. D. Finger, Z. Guo, C. Zer, and B. Shen. Functional regulation of FEN1 nuclease and its link to cancer. *Nucleic acids research*, 39(3):781–794, 2011.
- [200] S. Zielke and A. Bodnar. Telomeres and telomerase activity in scleractinian corals and *Symbiodinium* spp. *The Biological Bulletin*, 218(2):113–121, 2010.

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Appendix p

Appendix p1

Used Reagents, Chemicals, and Kits

Advantage Taq polymerase	BD, Heidelberg, Germany
BigDye® Terminator v1.1 Cycle Sequencing Kit	Applied Biosystems, Foster City, CA, USA
β-Mercaptoethanol	Sigma-Aldrich, Munich, Germany
Calcium acetate	Merck, Darmstadt, Germany
cDNA Rapid Library Preparation Kit for the GS FLX Titanium Series	Roche, Mannheim, Germany
Chloroform	Merck, Darmstadt, Germany
DC Protein Assay	BioRad, Munich, Germany
DNeasy Blood & Tissue Kit	Qiagen, Hilden, Germany
emPCR– Lib-L SV Kit for GS FLX Titanium Series	Roche, Mannheim, Germany
Eosin G-Lösung 0,5% wässrig	Roth, Karlsruhe, Germany
Ethanol	Merck, Darmstadt, Germany
Exonuclease I	Fermentas, St. Leon-Rot, Germany
Formaldehyde	Sigma-Aldrich, Munich, Germany
GoTaq polymerase	Promega, Mannheim, Germany
Haematoxylin	Roth, Karlsruhe, Germany
Isopropanol	Merck, Darmstadt, Germany
LE agarose	Biozym, Hessisch Oldendorf, Germany
NaCl	Merck, Darmstadt, Germany
NaOCl	AppliChem, Darmstadt, Germany
protease inhibitor cocktail tablet	Roche, Mannheim, Germany
QIAgen RNase-Free DNase	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
QIAshredder Mini Spin Column	Qiagen, Hilden, Germany

Appendix p

Quantitative Telomerase Detection Kit	US Biomax Inc., Rockville, MD, USA
RevertAid Premium First Strand cDNA Synthesis Kit	Fermentas, St. Leon-Rot, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Roti-Histokitt	Roth, Karlsruhe, Germany
Roti-Histol	Roth, Karlsruhe, Germany
Rotiphorese 10xTAE-Puffer	Roth, Karlsruhe, Germany
Shrimp Alkaline Phosphatase	Fermentas, St. Leon-Rot, Germany
SMARTer PCR cDNA Synthesis Kit	Clontech Laboratories Inc., Mountain View, CA, USA
SmartLadder MW-1700-10	Eurogentec, Seraing, Belgium
Sodium citrate	Sigma-Aldrich, Munich, Germany
SYBR Select Master Mix	Applied Biosystems, Foster City, CA, USA
SYBR Safe DNA gel stain	Invitrogen, Karlsruhe, Germany
TeloTAGGG Telomere Length Assay	Roche, Mannheim, Germany
TRI-reagent	Sigma-Aldrich, Munich, Germany
Wizard® SV Gel and PCR Clean-Up System	Promega, Mannheim, Germany
Xylol substitute (Xylene)	Merck, Darmstadt, Germany

Appendix p2

putative genes

Encountered putative genes in the combined_transcriptome with contig ID, expected domains, observed domains, E-value, contig length, and number of reads in contig are listed in the table on the following pages.

Category	Gene name	Contig ID	Domains expected	Domains observed	E-value ¹	Contig length (bp)	No. of reads in contig
DNA repair	APEX	14481	EEP, SAP	EEP=exo_endo_phos ^{2,3}	1E-144	2121	21
	CLUAP1	5706	Cluap1	Cluap1 ²	1E-160	1741	67
	DCLRE1A	40998	DRMBL, Lactamase_B	DRMBL ^{2,3}	2E-33	697	5
	LIG3	13556	zf_PARP, BRCT, OBF_DNA_ligase_family, Adenylation_DNA_ligase_like	mRNA_cap_enzyme ² , DNA_ligase_A_C ² , zf-CCHH ^{2,3} , Adenylation_DNA_ligase ³ , OBF_DNA_ligase_family ³	0.00	1526	23
	ERCC3	55184	Helicase_C_3,Ploop_NTPase	n.a.	5E-73	458	4
	LIG4	19291	DNA_ligase_IV, BRCT, OBF_DNA_ligase_family, Adenylation_DNA_ligase_like	BRCT ^{2,3} , DNA_ligase_IV ^{2,3} , DNA_ligase_A_C ² , OBF_DNA_ligase_family ³ , Adenylation_DNA_ligase	1E-132	1839	14
	MDC1	18215	BRCT, FHA	Adenylation_DNA_ligase_like ³			
	MRE11	20648	Mre_DNA_bind; mre11	2x BRCT ^{2,3}	1E-39	1306	15
	MSH3	26833	MutS_I,MutS_II, MutS_IV, P-loop_NTPase	mre11_dna_bind ^{2,3} , MUTSd ² , MutS_III ³ , MUTSd ² , ABC_ATPase ³	9E-31	1833	13
	PIF1	7511_s3	Herpes_Helicase, PIF1, UvrD_C_2, P-loop_NTPase, P-loop containing Nucleoside Triphosphate Hydrolases	MUTSd ² , ABC_ATPase ³ , DUF889 ² , AAA_30 ³	1E-107	1185	9
	PMS2	46661	MutL_C, HATPase_c, TopoII_MutL_Trans	DUF889 ² , AAA_30 ³	6E-16	2276	34
	POLB	22990	Rel_Spo_like	MutL_C ^{2,3}	2E-70	759	4
	Rad23	11341	XPC-binding, UBA, UBQ	POLXc ² , Rel-Spo-like ³	1E-82	859	11
	SF3B3	7952	CPSF_A, MMS1_N	STII ² , UBQ, UBA ^{2,3} , XPC-bin ³	7E-97	1332	29
	SPF45	3822	G-patch domain, RRM	CPSF_A ²	3E-65	1486	45
	TDPI	8109	zf-CCHH, FHA, PLDc_SF	G-patch domain, RRM ^{2,3} , Tyr-DNA-phospho ² , 2xPLDc_SF ³	1E-104	2053	102
					1E-165	2969	44

Category	Gene name	Contig ID	Domains expected	Domains observed	E-value ¹	Contig length (bp)	No. of reads in contig
	TOP3A	22003	TopA, zf_C4_Topoiso, zf_GRF ^{2,3} , TOP1Ac, zf_GRF, TOPRIM		1E-15	364	12
	XPC	22143	rad4, BHD3, Rad4, BHD1, BHD2	Rad4, BHD3 ^{2,3}	1E-102	1589	12
	XRCC5	2427_sl	Ku_PK_bind, Rnase_H, vWFA	Ku_PK_bind ^{2,3} , Ku78 ² , Ku_C ² , KU ³	2E-98	2151	88
	TEP1	28769	COG2319, TEP1_N, TROVE, DUF4062, WD40, P-loop_NTPase	TROVE ^{2,3}	3E-46	1029	8
		16057		TROVE ^{2,3}	3E-35	723	18
Apoptosis	Bcl-2	1117_s2	BH4, bcl-2, Bcl-2_like	BCL ²	2E-17	1602	330
	BAX-like	2467	bcl-2, Bcl-2_like	BCL ²	2E-36	920	160
	AIFM1	47148	Pyr-redox, Pyr-redox2	Pyr-redox ^{2,3}	7E-87	702	4
	BAX	17369	bcl-2, Bcl-2_like	BCL ² , Bcl-2_like ³	1E-16	920	160
		2467			3E-36	920	160
		47930			7E-09	492	4
	BCL2L1	5166	BH4, bcl-2, Bcl-2_like	BCL ² , Bcl-2_like ³	5E-13	1612	75
		3097			7E-20	1176	129
		44964			3E-15	574	5
		1117_s2			7E-17	1602	330
		1117_sl			1E-15	546	13
	AIFM3	2525	CoA_CoA_reduc, Pyr_redox, Rieske, NADB_Rossmann	Rieske ^{2,3} , Pyr_redox ^{2,3} , NAD_binding_8 ³	1E-152	2859	156
		10414		Pyr_redox_2 ² , Pyr_redox_3 ³	2E-90	1384	32
Autophagy	BECN1	17504	APG6, MT	APG6 ²	1E-143	1155	16
	ATG2	9723	MRS_6, ATG_C, Chorein_N, ATG2_CAD	ATG_C ²	1E-100	1404	35
	ATG12	483_s55	UBQ	APG_12 ² , UBQ ³	4E-39	1977	45
	ATG 8	16367	UBQ	PG_12 ² , UBQ ³	2E-54	851	18
	ATG4	55112	Peptidase C54	Peptidase C54 ^{2,3}	2E-67	500	4

Category	Gene name	Contig ID	Domains expected	Domains observed	E-value ¹	Contig length (bp)	No. of reads in contig
Oxidative stress	Ph4	16618	P4Hc, EF_hand_5, Efh	P4Hc ² , 2OG-FeII_Oxy_3 ³	3E-34	1572	17
other	Wnt-1	23506	WNT1, wnt	WNT1 ² , wnt ³	1E-39	757	11
	SIRT4	17855 + 110084	SIR2	SIR2 ^{2,3}	1E-117	1033	16
	SIRT1	6569	SIR2	SIR2 ^{2,3}	3E-39	2358	56
	AOX	3861	n.a.	AOX ² , Ferritin_like ³	1E-113	4211	101

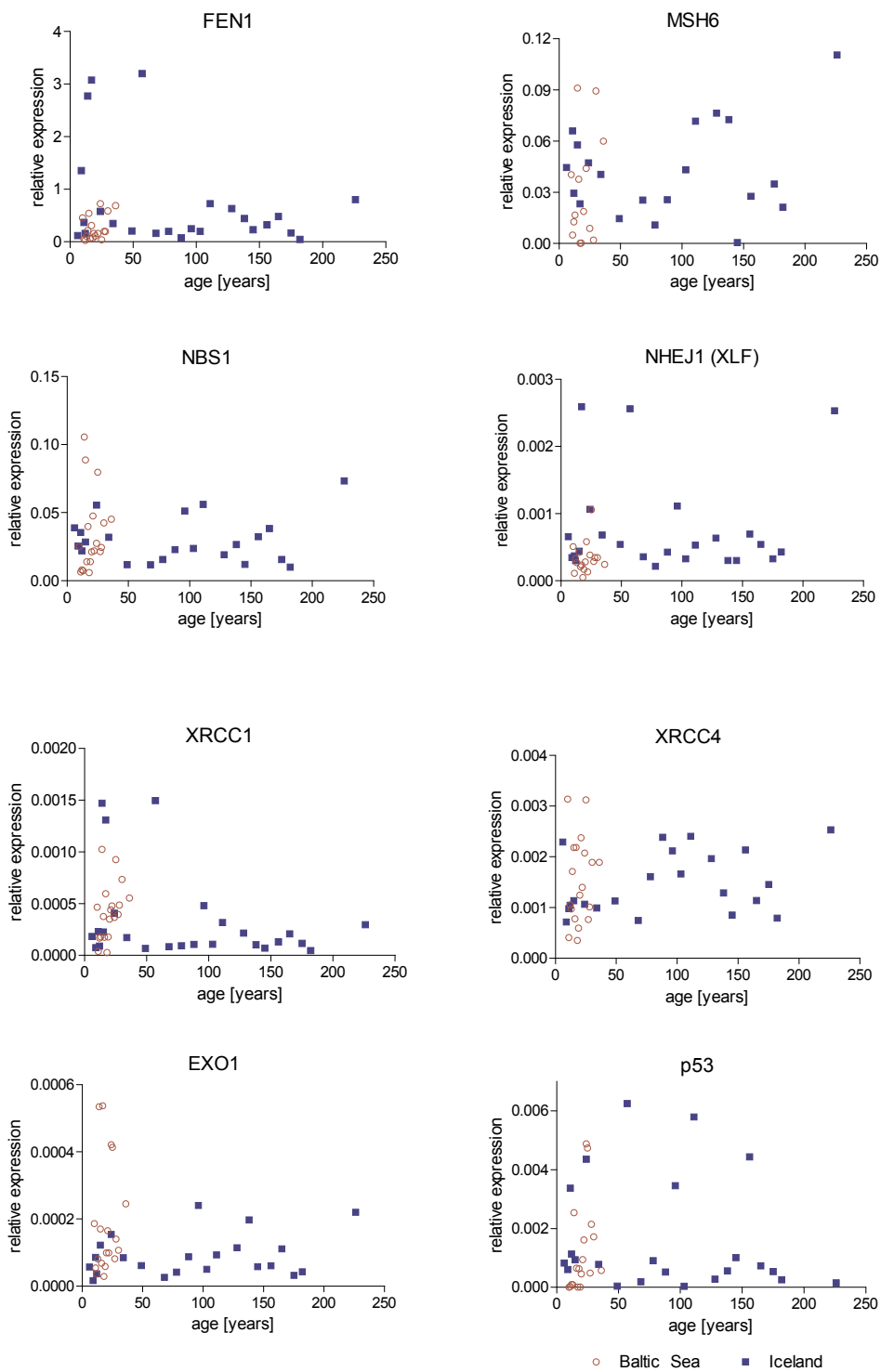
1: E-value taken from UniProtKB/Swiss-Prot.

2: Domains observed in PFAM via SMART (smart.embl-heidelberg.com).

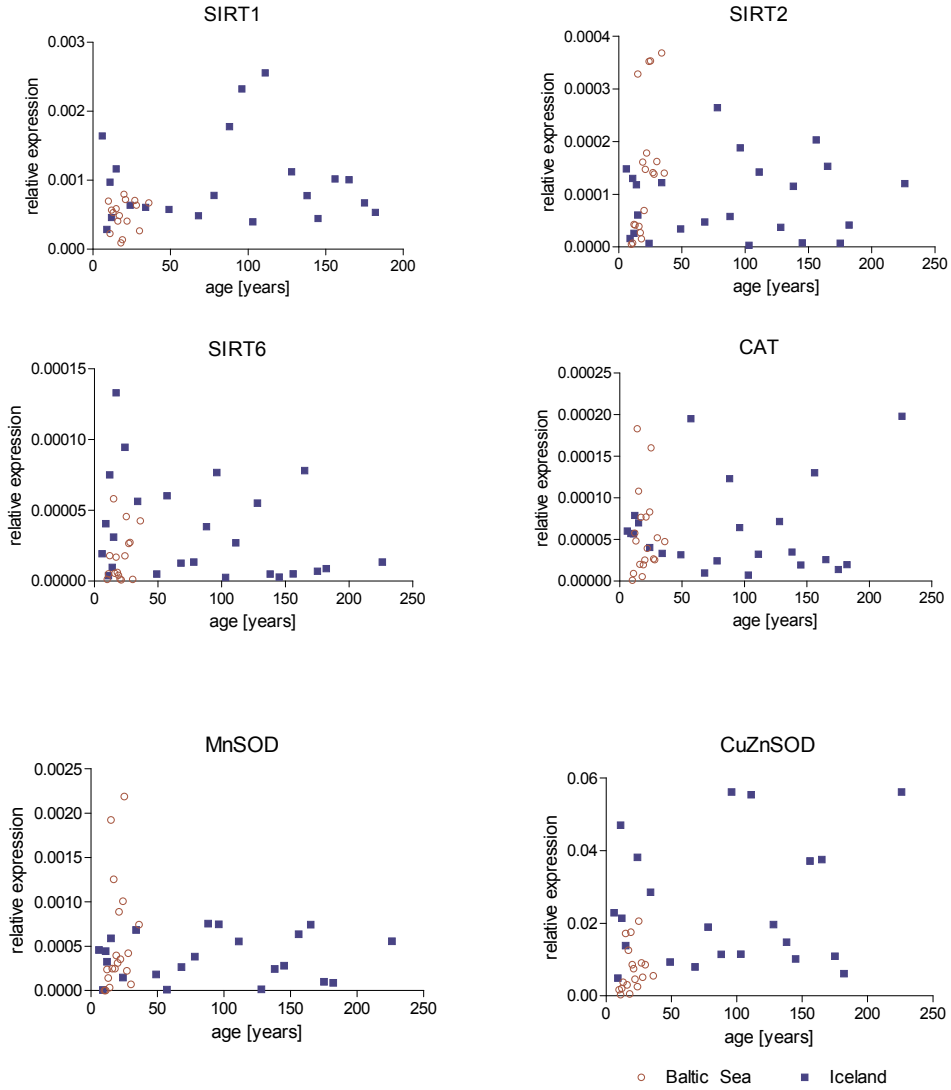
3: Domains observed in NCBI cDART (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>).

Appendix p3

gene expression



Appendix p



Relative realtime gene expression analyses of FEN1 (flap endonuclease 1), MSH6 (mutS homologue 6), NBS1 (Nijmegen breakage syndrome 1), NHEJ1 (Nonhomologous end-joining protein 1), XRCC1 (X-ray repair cross-complementing protein 1), XRCC4 (X-ray repair cross-complementing protein 4), EXO1 (exonuclease 1), p53 (Protein p53), SIRT1 (sirtuin 1), SIRT2 (sirtuin 2), SIRT6 (sirtuin 6), CAT (catalase), MnSOD (manganese superoxide dismutase), CuZnSOD (copper zinc superoxide dismutase) over age in the short-lived Baltic Sea and long-lived Iceland *A. islandica* populations.

Curriculum vitae

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EDUCATION

Jan 2010 - now Christian-Albrechts-University Kiel, Germany
PhD student in Biology

Research at the Institute of Clinical Molecular Biology
Title: "Investigation of the molecular ageing process of the long-lived bivalve *Arctica islandica*"

Oct 2007 - Dec 2009 University of Applied Sciences Bremerhaven, Germany
Master of Science in Bioanalytic

Research (03/09 - 12/09) at the Alfred-Wegener Institute for Polar and Marine Research in Bremerhaven, Germany
Thesis title: "Development of a vector construct for the transformation of the coccolithophore *Emiliana huxleyi*"

Research (10/08 - 02/09) at the technical transfer centre (ttz) in Bremerhaven, Germany

Project work (12/08 - 02/09) in cooperation with Impetus Biosciences

Project Title: "Optimization and Application of Ligase-dependent Probe Amplification (LPA) on crop"

Practical term (10/08 - 12/08) at the BIBIS

Project Title: "Prewrite for the development of a species discriminating assay based on ligase chain reaction (LCR)"

Sep 2000 - May 2004 University of Applied Sciences Mannheim, Germany
Bachelor of Science in Biotechnology (BSc with Honors)

Research (09/03 - 03/04) with Merck Chemicals Ltd. & British Biocell International in Cardiff, Wales

Thesis Title: "Development of the Internal Control reaction of a food hygiene lateral flow test"

Curriculum vitae

Research (08/02 - 01/03) at Newfound-Genomics in St. John's, Newfoundland, Canada
Project Title: "Validationstudy with 123 Probandns having Inflammatory bowl disease"

Sep 1989 - Jun 1998 Martin-Luther-Gymnasium in Rimbach, Germany
Allgemeine Hochschulreife (university entrance qualification)

Aug 1995 - Jun 1996 Royalton-Hartland Highschool in Middleport, New York, USA
Highschool Graduation

PUBLICATIONS & AWARDS

Philipp EER, Wessels W, **Gruber H**, Strahl J, Wagner AE, *et al.* (2012) Gene Expression and Physiological Changes of Different Populations of the Long-Lived Bivalve *Arctica islandica* under Low Oxygen Conditions. *PLoS ONE* 7(9): e44621 .doi:10.1371/journal.pone.0044621

Ungvari Z, Sosnowska D, Mason JB, **Gruber H**, *et al.* (2012) Resistance to Genotoxic Stresses in *Arctica islandica*, the Longest Living Noncolonial Animal: Is Extreme Longevity Associated With a Multistress Resistance Phenotype? *J Gerontol A Biol Sci Med Sci*. doi: 10.1093/gerona/gls193

Sep 2012 Poster: „Determining factors of maximum lifespan of the long-lived bivalve *Arctica islandica*“ Physiomar12 in Santiago de Compostela, Spain

Sep 2011 Poster: „Molecular and cellular investigations of the emerging ageing model organism: *Arctica islandica*“ RoSyBa Conference in Rostock, Germany

Mar 2011 Poster: „*Arctica islandica* as a model organism for ageing research: cellular, molecular and population investigations“ Excellence Cluster Retreat in Schleswig, Germany

Sep 2010 Poster: “Investigation of the molecular ageing process of the long-lived bivalve *Arctica islandica*” DZG Conference in Hamburg, Germany

Sep 2010 Posteraward 1st place in Developmental Biology at DZG conference in Hamburg, Germany

Dec 2009 University Funding Award 2nd place in Bremerhaven, Germany

Selbständigkeitserklärung

Hiermit versichere ich, Heike Gruber, dass die vorgelegte Arbeit nach Inhalt und Form die eigene Arbeit ist; diese Arbeit weder ganz noch zum Teil an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht wurde; diese Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

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Heike Gruber