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Mechanismen zellulärer Alterungsprozesse bei polaren und borealen marinen Evertebraten

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Strategies of cellular ageing in polar and temperate marine Invertebrates

Stiftung Alfred-Wegener-Institut
für Polar- und Meeresforschung
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Strategies of cellular ageing in polar and temperate marine Invertebrates

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USE YOUR HEALTH, EVEN TO THE POINT OF WEARING IT OUT. THAT IS WHAT IT IS FOR. SPEND ALL YOU HAVE BEFORE YOU DIE; AND DO NOT OUTLIVE YOURSELF. DO NOT TRY TO LIVE FOREVER. YOU WILL NOT SUCCEED

Shaw GB. The doctor's dilemma. London: Penguin, 1957:86-7.

CONTENTS

LIST OF SELECTED ABBREVIATIONS

ABBREVIATION	
ADP	adenosine diphosphate
AFDM	ash free dry mass
ATP	adenosine triphosphate
C	cytochrome c
COX	cytochrome c oxidase
CS	citrate synthase
e.g.	for example - *abbr. of "exempli gratia"* [Lat.]
GSH	reduced glutathione
GSSG	oxidised glutathione
H_∞	mean asymptotic shell height
HPLC	high performance liquid chromatography
H_t	shell height at age t (year)
i.e.	that is - *abbr. of "id est"* [Lat.]
k	growth constant of VBGF
MLSP	maximum life span
MP	membrane potential
N	number of individuals
Q	ubiquinone
R	respiration
RCR	respiratory control ratio
R_M	mitochondrial respiration
ROS	reactive oxygen species
S_∞	mean asymptotic shell size
SMR	standard metabolic rate
SOD	superoxide dismutase

S_t	shell size at age t (year)
t	time
t_0	age where shell size equals zero
tGSH	total glutathione (2 * GSSG + GSH)
VBGF	von Bertalanffy growth model

SUMMARY

Organisms show vastly different maximum lifespans, even when comparing closely related species of similar lifestyle. In marine ectotherms, a tendency has been perceived for higher MLSP in organisms living in permanently cold environments, compared to similar species from warmer waters. This raises the question how environmental temperature influences the ageing process in marine ectotherms. Antarctic waters are characterized by year round constantly low temperatures between -1.9 and +2.5°C, whereas species from temperate coastal waters can experience wide temperature fluctuations (0-18°C). Temperature has an impact on metabolic rates and, presumably, on the intensity of metabolic reactive oxygen species (ROS) formation in marine ectothermal species. According to the "Free Radical-Rate of Living" theory (Pearl, 1928; Harman, 1956), lower SMR and, therefore, lower generation rate of reactive oxygen species could be a prime factor responsible for a longer MLSP.

I investigated chronological and physiological ageing in differently aged individuals of Antarctic and temperate bivalve species: two burrowing mud clams, the Antarctic *Laternula elliptica* (Antarctic Peninsula, MLSP ~36 years) and the temperate *Mya arenaria* (North Sea, MLSP ~13 years), and two swimming scallops, the Antarctic *Adamussium colbecki* (Terra Nova Bay, MLSP ~45 years) and the temperate *Aequipecten opercularis* (Irish Sea, MLSP ~8-10 years), looking for physiological mechanisms that lead to higher maximum life spans of the Antarctic compared to temperate bivalve species. Comparing the physiological ageing in temperate mud clams and scallops, I also evaluated the effect of different motility lifestyles (swimming vs. burrowing) on the physiological ageing process.

Among the mud clams, the longer-lived Antarctic species displayed higher antioxidant capacities, a lower standard metabolic rate, and reduced rates of mitochondrial H_2O_2 generation, resulting in a slower decrement of mitochondrial functions and the conservation of tissue redox state with age. In the scallop group, a less pronounced decrease in mitochondrial and antioxidant enzyme activities and the avoidance of waste accumulation in the Antarctic scallop may account for the higher MLSP in the polar animal. SMRs of the two scallop species were not different throughout all ages.

Despite shorter MLSP and the active swimming lifestyle, mitochondria of the temperate scallop exhibit slower senescence than temperate mud clam mitochondria. This may indicate that the temperate scallop may conserve optimal physiological functioning and thus an active lifestyle as long as possible in order to evade predation and other interactive extrinsic mortality factors.

The results speak for a principle applicability of the “Free Radical-Rate of Living” theory to the ageing process in marine bivalves. The diverse mechanisms and strategies, which lead to the species and population specific MLSPs, indicate, that the absolute values and changes in the investigated physiological parameters are not simply based on environmental temperature regime and lifestyle (mobility), but are also influenced by other interactive parameters within the species specific ecological niche.

ZUSAMMENFASSUNG

Die maximale Lebenspanne (MLSP) von Organismen, selbst von nah verwandten Arten mit ähnlicher Lebensweise, ist von erstaunlich unterschiedlicher Dauer. Untersuchungen mariner wechselwarmer Tiere ergaben eine Tendenz zu höherer MLSP bei Tieren aus permanent kalten Lebensräumen als bei vergleichbaren Arten aus wärmeren Gewässern. Damit stellt sich die Frage nach dem Einfluss der Temperatur auf den Alterungsprozess mariner ektothermer Tiere. Antarktische Gewässer sind ganzjährig durch konstant niedrige Temperaturen zwischen -1.9 und +2.5°C charakterisiert, während Organismen aus borealen Küstengewässern vergleichsweise starken Temperaturschwankungen ausgesetzt sind (0-18°C). Temperatur beeinflusst die Stoffwechselraten mariner wechselwarmer Tiere und damit wahrscheinlich auch die Bildungsrate reaktiver Sauerstoffspezies. Gemäß der “Free Radical-Rate of Living” Theorie (Pearl, 1928; Harman, 1956) könnte ein niedriger Stoffwechsel und eine dadurch verringerte Bildungsrate reaktiver Sauerstoffspezies ein maßgeblicher Faktor sein, der zu einer hohen maximalen Lebenspanne führt. In der vorliegen Arbeit wurde die chronologische und physiologische Alterung antarktischer und borealer Muschelarten untersucht. Dies erfolgte an zwei grabenden Schlickmuscheln, der antarktischen *Laternula elliptica* (antarktische Halbinsel, MLSP ~36 Jahre) und der borealen *Mya arenaria* (Nordsee, MLSP ~13 Jahre), und an zwei schwimmenden Pilgermuscheln, der antarktischen *Adamussium colbecki* (Terra Nova Bay, MLSP ~45 Jahre) und der borealen *Aequipecten opercularis* (Irische See, MLSP ~8-10 Jahre). Weiterhin wurde die physiologische Alterung der borealen Schlickmuschel mit der Alterung der borealen Pilgermuschel verglichen, um den Effekt unterschiedlich aktiver Lebensweisen (grabend, schwimmend) auf den Alterungsprozess zu untersuchen.

Innerhalb der Schlickmuschel-Gruppe zeigte die langlebigere antarktische Art höhere antioxidative Kapazitäten, einen niedrigeren Standardstoffwechsel (SMR) und eine niedrigere mitochondriale H_2O_2 Bildung als die boreale Art. Dies führte zu einer langsameren Abnahme mitochondrialer Funktionen und zur Konservierung des Redoxverhältnisses im Gewebe mit zunehmendem Alter bei der antarktischen Art. In der Pilgermuschel-Gruppe könnten die geringere Abnahme mitochondrialer und antioxidativer Enzymaktivitäten und die Vermeidung der Anhäufung von Abfallprodukten mit dem Alter zu der längeren MLSP der antarktischen Art führen. Die Untersuchungen aerober Stoffwechselraten zeigten keinen generellen Unterschied zwischen beiden Pilgermuschelarten über die gesamte Alterspanne.

Im Vergleich der borealen Schlickmuschel und der Pilgermuschel wiesen die Mitochondrien der Pilgermuschel trotz kürzerer Lebensspanne und aktiverer Lebensweise

des Gesamtorganismus eine langsamere Alterung auf. Dies könnte darauf hindeuten, dass die Pilgermuschel eine zeitlebens optimale physiologische Fitness aufrecht erhält, um interaktiven äußeren Faktoren die zur Mortalität führen, z.B. Fressfeinden, möglichst lange zu entgehen.

Die Ergebnisse zeigen, dass die “Free Radical-Rate of Living” Theorie prinzipiell auf den Alterungsprozess von Muschen angewendet werden kann. Die Beobachtung unterschiedlicher Mechanismen und Strategien, die zu den jeweiligen MLSPs der verschiedenen Populationen führen, deutet darauf hin, dass die absoluten Werte und Änderungen der physiologischen Parameter mit dem Alter nicht nur auf die spezifische Anpassung an die Lebensraumtemperatur und die Lebensweise der verschiedenen Arten zurückzuführen ist. Weitere Faktoren, wie z.B. Interaktion mit anderen Organismen, können zusätzlich die MLSP der jeweiligen Population beeinflussen.

1. INTRODUCTION

1.1. What is ageing?

The process of ageing is defined as the progressive deterioration of cells, tissues and organs, associated with a decline in physiological function over time.

The question why and how we age has always fascinated researchers and up to now over 300 ageing theories were formulated. Some of the older theories have already been disapproved while others are still valid: the ancient Greeks stated that the secret of extending human life was an ascetic lifestyle, to maintain the flame of life without letting it flame too high. To date this would be called the “caloric restriction theory” and the underlying rationale has changed.

Most of today’s ageing theories can be grouped in two categories: Genetic / DNA related theories and cellular theories. Genetic / DNA related theories propose that the life span of an individual is strictly determined by genetic and DNA components e.g. longevity genes or by the shortening of telomeres, i.e. chromosome tails that have the same sequence of DNA bases repeated thousands of times and get shorter with each cell division until cell division can no longer occur. Cellular theories look at physiological processes in cells and organs and how they influence the life span of an individual. In contrast to the Gene / DNA group, the lifespan of individuals in cellular theories is not predetermined and can be modulated by environmental changes (e.g. temperature, food) which influence cellular physiological processes.

In the present study I focused on three cellular ageing theories which have the same origin. All three of them relate to the rate of aerobic metabolism and its metabolic by-products reactive oxygen species - **ROS**.

1.2. Three ageing theories with one origin

1.2.1. “Rate of Living” theory:

As an early ageing theory, the “Rate of living theory” was proposed by Raymond Pearl (1928). He noticed that species with high metabolic rate often have shorter life spans and proposed a negative correlation between standard metabolic rate (SMR) and maximum life span (MLSP). However the mechanistic link between metabolism and ageing remained unclear.

1.2.2.” Free Radical Theory of Ageing”

In 1956 Denham Harman developed the “Free Radical Theory of Ageing” based on irradiation experiments on *Drosophila melanogaster*. He proposed that ageing and

degenerative diseases associated with it, are caused by the endogenous generation of reactive oxygen species (ROS). The endogenous ROS generation was initially seen very controversial but supported when the cellular antioxidant enzyme superoxide dismutase was found. The only function of this enzyme is to destroy the reactive oxygen species superoxide anion.

Both theories, the “Rate of Living” theory and the “Free Radical Theory of Ageing”, are nowadays often used synonymously and can be combined to form the “Free Radical-Rate of Living” theory, as the generation of ROS is supposed to be positively correlated with SMR (Fleming et al., 1981).

However some species do not fall into the model-relationship of MLSP and SMR. Especially primates and birds life longer than deduced from their SMR (Perez-Campo et al., 1998). Careful analysis of the ROS production by mitochondria of these species demonstrated that they tend to produce fewer ROS than expected from their high SMR. A new view is that ROS generation is not strictly coupled to the oxygen consumption rate and that ROS production rather than SMR is correlated with MLSP (Ku et al., 1993; Barja et al., 1994b; Herrero and Barja, 1997b; Herrero and Barja, 1998).

1.2.3. „Uncoupling to Survive” hypothesis

The “Uncoupling to Survive” hypothesis (Brand, 2000) describes another facet of the “Free Radical-Rate of Living theory” and can supply an explanation where the ROS formation rate does not match with the level of SMR. It predicts that mitochondrial uncoupling mechanisms may modulate reactive oxygen species production, thereby uncoupling ROS production and oxygen consumption by mitochondria.

1.3. The nature of oxygen free radicals or reactive oxygen species (ROS)

An oxygen free radical is defined as any oxygen species that has one or more unpaired electrons occupying an atomic or molecular orbital by itself. In the context of the above definition, molecular oxygen is a di-radical as it contains two unpaired electrons. It is relatively inert and therefore not dangerous for the organism, due to the parallel spin of both unpaired electrons.

Other oxygen radicals are more reactive and always seek another electron to retrieve an electron pair. The biologically most relevant reactive oxygen species are the superoxide anion radical, hydrogen peroxide and the hydroxyl radical. While the superoxide anion and hydroxyl radical are classical radicals with one unpaired electron, hydrogen peroxide is not. These three and other oxygen species are therefore summarized under the term reactive oxygen species - **ROS**.

Starting from molecular oxygen, superoxide is formed by the addition of one electron. The addition of electrons to molecular oxygen or to the superoxide anion radical leads to hydrogen peroxide. Hydrogen peroxide is only mildly reactive but can diffuse through biological membranes and generate the most reactive and actually most dangerous and toxic hydroxyl radical, which is formed through the Fenton- reaction with transition metal catalysis.

ROS are produced by all aerobic organisms during normal metabolism. To cope with the generation of these hazardous species, antioxidant enzymes and small molecular antioxidants remove ROS and protect cells and their membranes and macromolecules from oxidation. The most important antioxidant enzymes are superoxide dismutase which catalyses the dismutation of superoxide and forms hydrogen peroxide, which is destroyed by catalase and glutathione peroxidase. Glutathione, vitamin E, vitamin C and coenzyme Q are examples for non- enzymatic small molecular antioxidants.

An imbalance of ROS generation and antioxidative defence can result in “oxidative stress”, an expression describing ROS-mediated deleterious processes such as oxidative damage of cell components like lipids, proteins and DNA.

1.4. Mitochondria and ROS

Mitochondria (Fig. 1.1) are regarded as a major source of reactive oxygen species as they are responsible for over 90% of the tissues’ oxygen consumption (Boveris and Chance, 1973; Staniek and Nohl, 2000). Hence it was suggested that mitochondria play a major role in the ageing process (Harman, 1972; Miquel, 1991; Barja, 1999). Sources of mitochondrial H_2O_2 are the enzyme monoamine oxidase, a constituent of the outer mitochondrial membrane, which catalyses the oxidative deamimation of biogenic amines and generates H_2O_2 on the cytoplasmic side of the membrane (Hauptmann et al., 1996), and the respiratory chain, located in the inner mitochondrial membrane (Fig. 1.2) (Barja, 1999; Cadenas and Davies, 2000; St-Pierre et al., 2002). The sites of ROS generation within the mitochondrial respiratory chain have been and still are subject to controversial discussion. Several studies identified two principal sites of oxygen radical formation of the respiration chain: complex I and complex III (Herrero and Barja, 1997b; Barja and Herrero, 1998; Barja, 1999; St-Pierre et al., 2002). In complex I, iron-sulphur centres or the flavin sites are assumed to be sources of oxygen radical generation (Herrero and Barja, 1997b; St-Pierre et al., 2002). In complex III of the mitochondrial

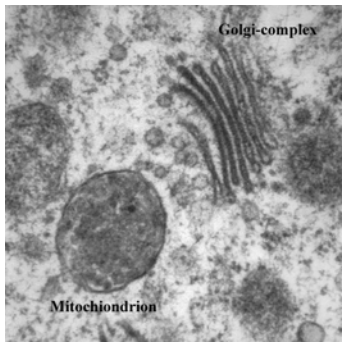

Fig. 1.1: Transmission Electron Microscope (TEM) image of a mitochondrion in mantle tissue from *L. elliptica.*

respiratory chain, redox-cycling ubiquinones have often been suggested to account for mitochondrial ROS generation. According to Nohl (1994) this does not happen under normal conditions, as the ubiquinone operates in an aprotic phase of the phospholipid layer and therefore a leak of electrons to molecular oxygen is not possible due to thermodynamic considerations. However, under certain conditions, e.g. mitochondrial membrane alteration due to cholesterol and erucic acid (long-chain fatty acid) insertion, single electrons leak from the ubisemiquinone to dioxygen, resulting in the formation of superoxide radicals (Gille and Nohl, 2001).

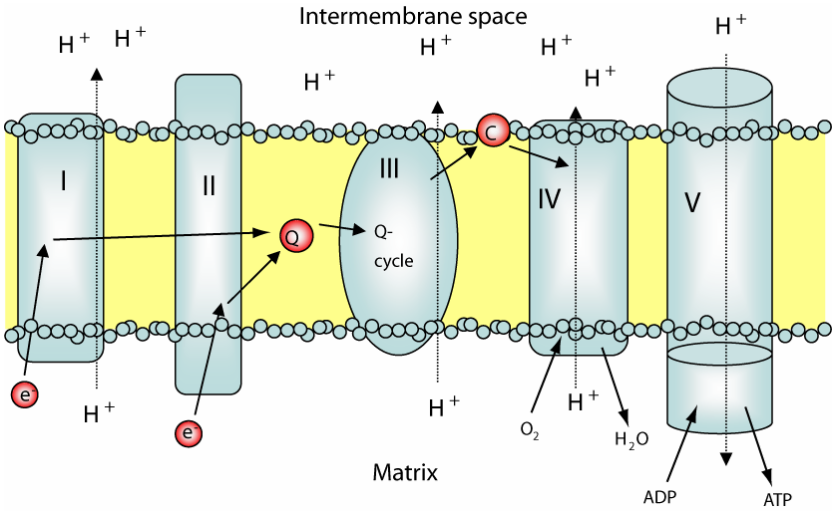

Fig. 1.2: Schematic overview of the components of the respiratory chain, situated in the inner mitochondrial membrane.

The ROS generation rate of the different formation sites is dependent on the tissue and the physiological state of the mitochondria (Barja, 1999). Mitochondria respiring with substrate alone (functional states 2, 4) normally generate higher amounts of oxygen radicals (nmoles H_2O_2/min/mg protein) than after ADP addition (state 3) where ROS generation is nearly absent (Loschen et al., 1971; Herrero and Barja, 1997a; Li et al., 1999). This is attributed to the high reduction state of the respiratory chain components under state 2 and 4 conditions (Barja, 1999; Cadenas and Davies, 2000). However, this clear-cut relationship can not always be taken for granted as e.g. mitochondria isolated from rat and pigeon heart and brain respiring with succinate showed H_2O_2 generation rates as expected (higher H_2O_2 generation rate without and lower with ADP), but during pyruvate/malate respiration similar or even higher rates of H_2O_2 generation were found with ADP added than without (Herrero and Barja, 1997a). This indicates complex I as the main free radical generator in state 3. The percentage of consumed oxygen converted to ROS is, however, always lower under state 3 than state 2 and 4 conditions.

1.5. Proton leak

Another factor that might modulate mitochondrial ROS generation and has recently come into focus is the mitochondrial proton leak. The proton leak describes the non-phosphorylating reflux of protons from the mitochondrial intermembrane space into the matrix, bypassing the ATPase. As stated above, ROS are normally generated to a greater extent under conditions where the respiratory chain components are in a highly reduced state, equivalent to high membrane potential. According to Brand (2000) and Skulachev (1998) the proton leak might prevent extensive ROS generation in such situations, by lowering the proton motive force and lessen the reductive tension. According to Porter (1996) the proton leak can be altered by the inner mitochondrial membrane surface area (cristae density) and the level of unsaturated fatty acids in the mitochondrial membrane. The nature of the proton leak is, however, still unclear. Protons probably leak through the membrane bilayer as it exists, or may be funnelled by proteins like uncoupling proteins (UCP) (for review see Brand et al., 1999; Brand, 2000).

About 80% of the ROS generated by mitochondria are destroyed by antioxidants like SOD or catalase, but the remaining 20% escape detoxification and lead to lipid, protein and DNA damage (Nohl and Hegner, 1978). Mitochondrial components are supposed to be a major target of reactive oxygen species, as they are in close vicinity of the generation site (Shigenaga et al., 1994; Barja, 1999). Mitochondrial DNA for example, suffers about 10-times more from oxidative damage than nuclear DNA (Shigenaga et al., 1994).

Next to mitochondria, endogenous reactive oxygen species can be also formed by exogenous environmental conditions like ultraviolet radiation, x-rays and toxic chemicals. However, while these factors may secondarily modify lifespan of cells and individuals, the principal process of ageing is related to routine rates of ROS production in normal metabolism. The resulting MLSP can differ enormously, even between related animal species and between those which display similar levels of performance (Fig. 1.3).

Species	MLSP
Yeast	2-4 days
Nematode (*Caenorhabditis elegans*)	30
Fruitfly (*Drosophila melanogaster*)	60
Short-Tailed field vole (*Microtus agrestis*)	1.8 years
Pacific salmon (*Onchorynchus*)	3-6
Mouse (*Mus musculus*)	4.2
Earthworm (*Lumbricus terrestis*)	6
American hookworm (*Necator americanus*)	15
Herring gull (*Larus argentatus*)	41
Lowland gorilla (*Gorilla gorilla graueri*)	47
Thick stemmed bamboo (*Phyllostachys bambusoides*)	120
Humans (Jeanne Calment)	122
Warty oreo (*Allocyttus verrucosus*)	>130
Rockfish (*Sebastes aleutianus*)	140
Pinnapple related (*Puya raimondii*)	150
Tortoise (*Geochelone gigantea*)	150
Sturgeon (*Acipenser fulvescens*)	152
Freshwater pearl shell (*Margaritifera margaritifera*)	190
Ocean quahog (*Arctica islandica*)	220
Great Basin bristlecone pine (*Pinus longaeva*)	4862
Hydra	non-ageing

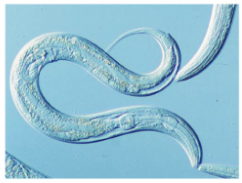

Fig. 1.3: Overview over the vast difference in maximum life span (MLSP) found in animals and plant species.

1.6. How oxygen radicals relate to ageing: current knowledge

Up to now, mitochondrial oxygen radical generation, cellular redox parameters and the change of antioxidant capacity and oxidative damage with age i.e. the relationship between chronological and physiological age, have been mainly investigated in terrestrial mammals, birds and invertebrates (Sohal et al., 1987; Sestini et al., 1991; Leeuwenburgh et al., 1994; Goodell and Cortopassi, 1998; Lopez-Torres et al., 2002). The results gained from these studies are quite diverse and do not always reflect a consistent picture. While the relationship of mitochondrial oxygen radical generation or antioxidant capacity and chronological age is still under discussion, there is good evidence for an age-related increase in oxidative damage of lipids, proteins and DNA (Shigenaga et al., 1994; Perez-Campo et al., 1998; Barja, 1999; Barja, 2004).

In aquatic organisms the majority of studies investigating oxidative stress parameters focused on effects of environmental parameters like temperature or heavy metal contaminants (Viarengo et al., 1989; Viarengo et al., 1995; Buchner et al., 1996; Regoli et al., 1997; Regoli et al., 2000; Estevez et al., 2002; Regoli et al., 2002; Heise et al., 2003; Abele and Puntarulo, 2004; Keller et al., 2004). Ageing studies mostly focused on growth and the identification of the chronological age (years), (Brey et al., 1995; Ziuganov et al., 2000; Cailliet et al., 2001; La Mesa and Vacchi, 2001). Only a few studies investigated the relationship between chronological age and oxidative stress parameters, as a measure for physiological fitness, i.e. addressed the relationship between chronological and

physiological age (Viarengo et al., 1989; Buchner et al., 1996; Canesi and Viarengo, 1997; Zielinski and Pörtner, 2000; Sukhotin and Pörtner, 2001; Sukhotin et al., 2002; Correia et al., 2003; Heilmayer et al., 2003) (Appendix). Like in the studies of terrestrial vertebrates and invertebrates, results are quite diverse. Here again a uniform trend of antioxidant capacity with age was not found but oxidative damage generally increased with age in marine ectotherms. Information about changes in mitochondrial functions with age has been completely lacking in marine ectotherms.

1.7. Why study the ageing process in marine bivalves?

Laboratory analyses in the two established terrestrial model invertebrates for ageing, *Drosophila melanogaster* and *Caenorhabditis elegans*, revealed a negative correlation between temperature and MLSP (Miquel et al., 1976; Hosono et al., 1982; Sestini et al., 1991). In the world's oceans marine ectotherms are distributed over a wide temperature range from -1.9 to +30°C and have adapted to widely different temperature regimes over evolutionary times. With respect to MLSP, marine ectotherms show a somewhat similar picture as *D. melanogaster* and *C. elegans*: an increase in MLSP from warmer towards permanently cold waters (Brey, 1991; Brey et al., 1995; Ziuganov et al., 2000; Cailliet et al., 2001; La Mesa and Vacchi, 2001). However, these latter data were not obtained in laboratory experiments but elaborated from field samples collected from warm to cold water environments.

This shows that colder temperatures must slow down physiological ageing and that the link may be lower metabolic rates and correspondingly lower rates of reactive oxygen species generation from aerobic mitochondria at low temperature.

However, comparative studies show that mitochondrial ROS formation is not always a linear function of respiration rate (Ku and Sohal, 1993; Sohal et al., 1993; Barja et al., 1994b; Brunet-Rossinni, 2004). Moreover, when investigating cold and warm adapted marine ectotherms, several species in the cold show mitochondrial adaptations like higher mitochondrial densities, higher mitochondrial cristae density and altered membrane fatty acid composition, which allow to maintain physiological functions at reduced environmental temperatures (Archer and Johnston, 1991; Johnston et al., 1994; Porter et al., 1996; St.-Pierre et al., 1998; Guderley and St-Pierre, 2002; Sommer and Pörtner, 2002). Such modifications may modulate the free radical leakage from mitochondria and therefore compromise the straight relationship between SMR and MLSP

Marine ectotherms from different temperature regimes therefore represent ideal model organisms to investigate cellular ageing theories based on metabolic rates and mitochondrial oxygen radical formation. In the group of marine ectotherms, especially

bivalves are very attractive organisms for ageing studies as they are characterized by largely different habitats and lifestyles and their shells provide a record of their chronological age. The investigation of species from within one class, but with different lifestyles, as displayed by rather inactive benthic burrowing clams in contrast to active swimming scallops, might further provide insight into ageing strategies depending on lifestyle. In contrast to other classical ageing-models like rats, mice, *D. melanogaster* and *C. elegans* it is possible to compare animals from different climatic environments in the wild and determine their individual chronological age and natural MLSP at each location.

1.8. Aims of this study

The objective of this study was to investigate chronological and physiological ageing patterns in marine bivalves under *in situ* conditions and to examine the modulating effects of environmental temperature and lifestyle on these patterns. Physiological parameters with respect to the accelerating effect of temperature and physical activity on metabolic rates and reactive oxygen species (ROS) formation were investigated in burrowing and swimming polar and temperate marine bivalves with different maximum lifespans. The study aims at the identification of physiological principles underlying the vastly differing MLSPs of the investigated bivalve species.

Three aspects were specifically addressed:

(i) Does the comparison of changes in oxygen-related physiological parameters with age in marine bivalve species with different maximum lifespans point towards an involvement of oxygen radical species in the physiological ageing process and can therefore the “Free Radical – Rate of Living” theory be applied?

(ii) Are the higher MLSPs in cold adapted than in temperate marine bivalves the result of a mere temperature dependent slow down of a uniform ageing process, or do we find different ageing patterns in the investigated species which relate to biochemical adaptations to the species specific environmental temperature regimes?

(iii) Do bivalve species with high and low activity lifestyles follow a common ageing pattern at different rates, or does an active and energy consuming lifestyle lead to completely different strategies with respect to maintenance of physiological fitness and onset of senescence with age.

2. MATERIAL AND METHODS

2.1. Investigated species

In the present work, two burrowing benthic mud clam species, *Laternula elliptica* from Antarctica (King George Island) and *Mya arenaria* from the North Sea (Netherlands), and two epibenthic swimming scallop species, *Adamussium colbecki* from Antarctica (Terra Nova Bay) and *Aequipecten opercularis* from the Irish Sea (around Isle of Man) (Fig. 2.1A and B), were investigated to study the influence of environmental temperature (temperate vs. polar) and lifestyle (burrowing vs. swimming) on the physiological ageing process. All four species represent key species from their specific environment. In the following I present general information on the investigated species.

Fig. 2.1A: Sampling stations of *L. elliptica* and *A. colbecki.*

Fig. 2.1B: Sampling stations of *M. arenaria* and *A. opercularis.*

2.1.1. Mud clams

Mya arenaria:

a short- lived, burrowing benthic mud clam from the North Sea

Mya arenaria (*Myoida,* Fig. 2.2), also called s*oft-shelled clam* or *sand gaper,* is a filter feeding clam which burrows up to 25 cm deep into the sediment (Zwarts and Wanink, 1989). The clam has a wide distribution in the Northern hemisphere, on both Atlantic coasts as well on the Pacific east coast (Strasser, 1999). In America and Europe *M. arenaria* is known since the Pliocene. In Europe it became extinct at the beginning of the Pleistocene and is thought to have been reintroduced to European coastal zones in the 16th or 17th century. However, there is some evidence that the Vikings imported this species to Europe as early as 1245 (Petersen et al., 1992).

Fig. 2.2: the North Sea mud clam *M. arenaria.*

M. arenaria is a relatively large bivalve which can exceed 140 mm shell length. A maximum life span (MLSP) of 10-12 years is found on the Atlantic East coast (Brousseau, 1978; Strasser, 1999) whereas at the White Sea the maximum age is 18 years (A. Sukhotin pers. comm.) and in the Bay of Fundy (Maine) 28 years have been estimated (MacDonald and Thomas, 1980). *M. arenaria* is dioecious and has planktrotrophic larvae. First reproduction usually occurs at a shell size of 20 to 50 mm which corresponds to an age of 1-4 years, depending on growth rate (Strasser, 1999).

The species is a dominant member of the benthic infauna and occurs in densities up to 115 ind. m^{-2} (Warwick and Price, 1976; Winther and Gray, 1985; Strasser, 1999). It is an important food source for numerous species in benthic ecosystems, as well as for man. The most important predators of juvenile *M. arenaria* are crabs (e.g. the green crab *Carcinus maenas*), shrimp, shorebirds, nereids, nemertineans and flatfish (*Pleuronectes platessa*, *Platichtys flesus*). Adults are preyed upon by crabs, oystercatchers (*Haematopus ostralegus*) and curlew (*Numenius arquata*) and wintering sea ducks in the Baltic Sea (Emerson et al., 1990; Strasser, 1999). In North America the soft-shell clam has an important value for commercial fisheries (Strasser, 1999).

For the present study *Mya arenaria* were sampled in summer 2002 and 2003 with a box corer at a shallow subtidal muddy site close to Harlingen (53°09`N, 05°19 ´E), The Netherlands. Sampling depth was 2.75 m under sea level, which is about 1.80 m under low-water spring level. In the sampling area *M. arenaria* experiences large seasonal fluctuations in seawater temperature from -1 to +20°C.

Laternula elliptica:

a long-lived, burrowing benthic mud clam from South Shetland Islands, Antarctica

Laternula elliptica (*Pholadomyoida*, King and Broderip, 1831, Fig. 2.3) is one of the most abundant bivalves of the Antarctic and forms dense beds of up to 200 ind. m^{-2} (Wägele and Brito, 1990). The clam has a long history in Antarctic waters, first records of *L. elliptica* are from the late Pliocene from the Northern Antarctic Peninsula (Soot-Ryen, 1952; Jonkers, 1999). *L. elliptica* is found in water depths from 3-320 m (Powell et al., 2001) and is widely distributed in nearshore waters around the Antarctic continent and islands (Powell, 1965).

Fig. 2.3: The Antarctic mud clam *L. elliptica.*

L. elliptica is a relatively large bivalve with a maximal shell length > 100 cm, while other Antarctic bivalves measure less than 20 mm (Urban and Mercuri, 1998), and has a maximum lifespan of approx. 36 years (this study and T. Brey pers. comm.).

Like *M. arenaria, L. elliptica* is a filter feeding clam which burrows up to 50 cm deep into the sediment (Hardy, 1972). It appears to occupy a similar ecological niche as the genus *Mya* in the temperate Northern Hemisphere (Ralph and Maxwell, 1977). *L. elliptica* is a simultaneous hermaphrodite with lecithotrophic larvae and reaches maturity at about 50 mm (Bosch and Pearse, 1988 and G. Mercuri pers. comm.). In contrast to other *Laternulidae*, *L. elliptica* is able to rebury into the sediment when removed (Peck et al., 2004). This might be crucial for survival of this species as especially in the shallower areas clams can get uncovered from sediment by scouring icebergs. When uncovered, *L. elliptica* is often consumed by the sea stars *Odontaster validus* and *Cryptasterias turqueti*, the nemertean *Parborlasia corrugatus,* the gastropod *Neobuccinum eatoni*, the fish *Trematomus bernacchii* and the isopod *Glyptonotus antarcticus* (Wägele and Brito, 1990).

Laternula elliptica of the present study were collected by scuba divers in Potter Cove, King George Island, South Shetland Islands (62°14`S, 58°40`W) in 5 to 10 m water depth in November-February 2002/2003. In contrast to *M. arenaria*, *L. elliptica* of the Potter Cove experiences only small temperature fluctuations throughout the year from approx. -2 to +1.5°C (Alder and Centurion Araujo, 2003).

2.1.2 Scallops

Aequipecten opercularis:

a short-lived, actively swimming epibenthic scallop from the Irish Sea

Aequipecten opercularis (Linnaeus, 1758, Fig. 2.4), also called *queen scallop* or *queens*, belongs to the pectinid family which, together with the cephalopods, represent the only molluscs which developed swimming ability by means of jet propulsion. The scallop has a wide geographical distribution on the European continental shelf from Northern Norway to Gibraltar and within the Mediterranean (Waller, 1991). Maximum lifespan of *A. opercularis* is about 8-10 years with a maximum shell height of about 90 mm. Food uptake is accomplished by filter feeding. The animal is hermaphroditic with a gonad divided into a testis (proximal) and ovary (distal). Young *A. opercularis* are often attached by use of byssus while older individuals lie freely on the sediment (Ansell et al., 1991). The species has a high commercial value and is fished e.g. in the north Irish Sea around the Isle of Man. *A. opercularis* are active swimmers and sensitive to

Fig. 2.4: The Irish Sea scallop *A. opercularis.*

disturbance. Danger is sensed over astonishing distances so that a swimming escape reflex is initiated up to 1.5 m away from approaching divers or fishing gear (Chapman, 1981). Around the Isle of Man *A. opercularis* damaged by fishing gear, but also undamaged *A. opercularis*, are preyed upon by starfish *Asterias rubens*, crustaceans *Pagurus* spp., *Cancer pagurus* and the demersal fish *Callionymus lyra* (Veale et al., 2000a).

For the present study *Aequipecten opercularis* were sampled from the Irish Sea in August-September 2003 with a toothed-dredge around the Isle of Man, 15 miles south of Port St Mary, at 65 m water depth. In the Port Erin Marine Laboratory the scallops were kept in flow through aquaria provided with water directly from the sea at approx. 10°C until processed. Around the Isle of Man *A. opercularis* experiences seasonal temperature fluctuations from 7 to 14°C (K. Kennington and T. Shammon pers. comm.).

Adamussium colbecki:

a long-lived, actively swimming epibenthic scallop from Terra Nova Bay, Antarctica

Like *A. opercularis,* the Antarctic *Adamussium colbecki* (Smith, 1902, Fig. 2.5) belongs to the actively swimming pectinids and is one of the most common bivalves of the Antarctic shallow water benthos of the Ross Sea (Ansell et al., 1998). It has a circumpolar distribution at depths from 0 to 1.500 meters (Dell, 1972). The Antarctic scallop is thought to be an “old” species which reinvaded the shallow waters around Antarctica after the Holocene deglacation (Berkman, 1990). It is one of the largest species of the Antarctic bivalve fauna with up to 108 mm shell height (Berkman, 1990) and lives in dense beds of up to 60 ind. m^{-2} (Cattaneo-Vietti et al., 1997). Maximum age of *A. colbecki* has been previously considered to be 20 years (Berkman, 1990), however growth curves of the species are quite variable (Heilmayer et al., 2003) and the species is nowadays suspected to reach 45 years (Heilmayer pers. comm.) or even live over a century (Berkman et al., 2004). *A. colbecki* is dioecious and reaches maturity at a shell length of approx 40-50 mm. Young individuals are attached to the adults by byssus until they reach maturity and then change to a free living epibenthic lifestyle. *A. colbeck*i is an active swimmer with a level of performance comparable to temperate scallops (Ansell et al., 1998; Bailey et al., 2003). The ecological function of swimming for *A. colbecki* might be escape from predators like the fish *Trematomus bernacchii* and invertebrates (*Neobuccinum eatoni, Paraborlasia corrugata*)

Fig. 2.5: The Antarctic scallop *A. colbecki.*

(Vacchi et al., 2000) but also the avoidance of unfavourable conditions like scouring icebergs.

For the present study *Adamussium colbecki* were sampled in Terra Nova Bay ("Road Bay", 74°43`S, 164°13 ´E) in February 2004 at 40-70 m water depth. Water temperature in Terra Nova Bay varies from winter minimum -1.9 to a summer maximum of +1°C (Manzella et al., 1999; Povero and Petrillo, 2000). The animals were sampled by dredge from the RV "Italica" and either directly sacrificed after collection and frozen as separate tissues in liquid nitrogen, or frozen as a whole animal.

2.2. Methods

Different methods were used to determine the chronological and physiological age of the investigated bivalve species (Fig. 2.6). The techniques used for chronological age

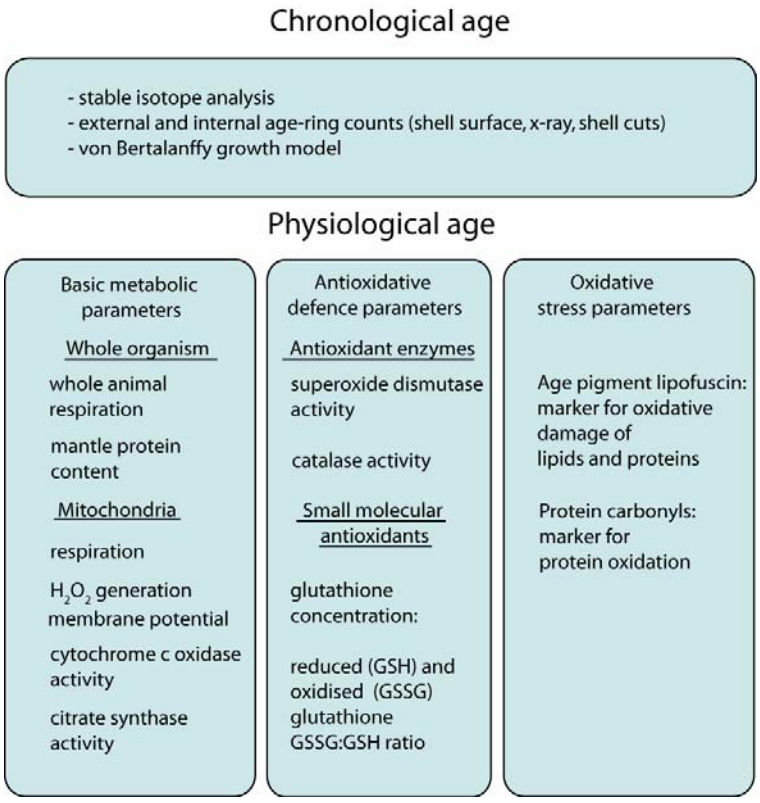

Fig. 2.6. Diagram showing the different approaches and techniques to investigate the chronological and physiological age of the investigated bivalve species.

determination and a model calculating lifetime respiration of the investigated bivalves will be described in more detail. The methods for measurements of physiological age parameters have been described in publications I-III and are only briefly summarized here.

2.2.1. Chronological age determination

Background

Lifelong growing hard structures such as mollusc shells or fish otholids can be used as records of individual chronological age if internal or external growth checks are formed at regular intervals in time. The formation of such growth checks is based on cyclic changes of environmental factors like temperature or food (for review see Richardson, 2001). In some bivalves, external growth checks can be identified reasonably well by eye on the outside of the shell surface (Newcombe, 1936; Feder and Paul, 1974; Evans and Tallmark, 1977; Brousseau, 1979; Winther and Gray, 1985; Allison, 1993; Sukhotin et al., 2003). However, in other species identification of external growth checks is impossible and disturbance marks may further complicate the identification of the "real" growth check pattern. In such cases internal growth mark patterns are more reliable to determine the individual age. Such patterns are analysed in polished dorso-ventral shell sections, either in the umbo and/or on the whole section from the umbo to the shell margin (MacDonald and Thomas, 1980; Brousseau and Baglivo, 1987) or in acetate peels of such sections (Richardson et al., 1979). The time interval of growth check formation, daily, monthly or annually, can be deduced either from mark recapture experiments (Brousseau, 1979; Chiantore et al., 2003) or from stable isotope analysis (Krantz et al., 1984; Brey and Mackensen, 1997; Heilmayer et al., 2003; Ivany et al., 2003). Stable isotope analysis over the growth axis of accreting organisms is a tool previously used in paleoenvironmental studies, as the shells contain records of seasonal and interannual climate variables which can be determined in fossil shell deposits from ancient times (Hickson et al., 1999). The isotopic composition of the carbonate in marine bivalve shells is mainly dependent on the isotopic composition of the surrounding seawater, with the $^{16}O/^{18}O$ ratio reflecting temperature and $^{12}C/^{13}C$ ratio reflecting primary production at the time of shell formation (Krantz et al., 1987; Wefer and Berger, 1991; Schöne, 2003). This technique is increasingly employed in the investigation of living accreting organisms like bivalves, as biological variables like age and growth is contained in theses records (Ivany et al., 2003). In the investigated bivalve species of the present study the annual deposition of growth checks was verified by isotope analysis for *L. elliptica, A. opercularis* and *A. colbecki* in previous studies (Brey and Mackensen, 1997; Heilmayer et al., 2003; Heilmayer et al., 2004b) and for *M. arenaria* in the present study.

Age determination of the investigated bivalves

To determine the individual age within the different species of the present study, different techniques of age determination were used and modified for application to species specific shell and growth structure. For *M. arenaria,* age determination was developed in cooperation with Dr. Olaf Heilmayer (Florida Atlantic University). Isotope profiles of carbonate samples taken along the dorso-ventral transect of the outer shell layer of 4 individuals were compared with corresponding growth checks visible on the outer shell surface or in x-ray photographs to distinguish between annually formed growth marks and disturbance marks. The distance of all identified annual growth to the umbo was measured in 23 individuals and a von Bertalanffy growth function (VBGF) was fitted to the resulting 181 size-at-age data using the nonlinear iterative Newton algorithm (Publ. I and III):

$S_t=S_\infty*(1-e^{-k*(t-t0)})$

where S_t is shell size at time t, S_∞ mean asymptotic shell size (i.e. the constant shell size the animal will reach after growth stops), k is the growth constant, and t_0 is the time at which theoretically shell size is zero.

Heilmayer et al. (2004b) confirmed that shell growth checks in *A. opercularis* from the English Channel are deposited annually and hence this was assumed to be true for *A. opercularis* from the Isle of Man, too. A VBGF was fitted to 276 size-at-age data. 76 animals were aged in this study and 200 additional data were kindly provided by E. Allison (Allison 1993), who worked at the same location (Publ. III). For *A. colbecki* size-at-age data provided by Heilmayer et al. (2003) were used to establish a VBGF. Note that in scallops, shell height is used for size whereas in clams usually shell length is used (Fig. 2.7).

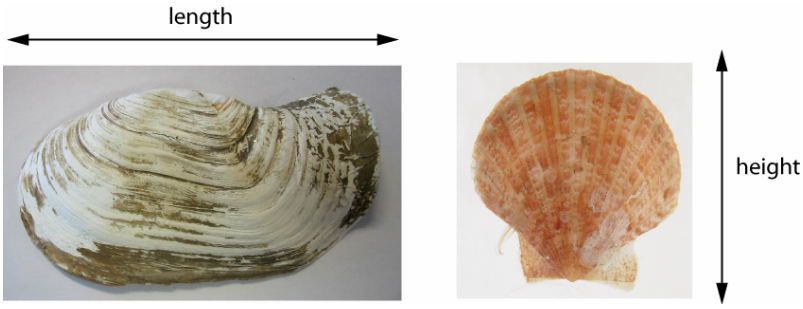

Fig. 2.7: Length and height determination of a mud clam and scallop.

To estimate age t from size S_t in *M. arenaria*, *A, opercularis* and *A. colbecki*, we fitted an inverse VBGF to the same size-at-age data:

age $t = \ln (1 - S_t / S_\infty) / -k + t_0$

In *L. elliptica* the complex shell surface structure does not allow for reliable age estimates. Therefore individuals were aged by counting the annually formed growth checks (Brey and Mackensen, 1997) in polished cuts through the shell umbo (Fig. 2.8).

Fig. 2.8: Polished cut through the umbo of an *L. elliptica* individual. The arrows point toward winter growth checks. Photo by M. Voigt.

2.2.2. Physiological age determination

Basic metabolic parameters:

- Standard Metabolic Rate

In *M. arenaria*, *L. elliptica* and *A. opercularis* whole animal standard metabolic rates (SMR) were determined (Publ. I and III) as oxygen consumed per individual ash free dry weight at species specific environmental mean temperature (Tabl. 2.1).

Tabl. 2.1: Water temperature range at sampling station of the investigated bivalves species.

species	Sampling location	Temperature range Min ---> Max °C	Annual mean temperature °C
M. arenaria	North Sea, Netherlands	-1 to +20	~10
L. elliptica	King George Isl., Antarctica	-2 to +1.5	~0
A. opercularis	Irish Sea, Isle of Man	+7 to +14	~10
A. colbecki	Terra Nova Bay, Antarctica	-1.9 to +1	~0

Source of water temperature information: M.a.: National Institute for Coastal and Marine Management / RIKZ; L.e.: Alder et al., 2003; A.o.: K. Kennington and T. Shammon (Port Erin Marine Laboratory) pers. comm.; A.c.: Manzella et al. 1999; Povero et al. 2000.

Oxygen consumption of differently aged individuals of all bivalve species was determined following the same protocol. Prior to the experiment the animals were left unfed over at least 2 days in order to reduce the impact of specific dynamic action (SDA). In the experimental setup the animals were allowed to accommodate to the respiration chambers over night before the measurements were started. The oxygen consumption was measured in an intermittent flow system with oxygen microoptodes. A detailed description of the measurement procedure is given in publication I and III.

In the case of *A. colbecki,* SMRs were determined in a previous study by Heilmayer and Brey (2003) using a similar protocol.

- Lifetime Aerobic Turnover calculated by a model

To investigate the relationship between maximum lifespan (MLSP) and oxygen consumption rate, respectively the lifetime aerobic turnover (i.e. the accumulated aerobic consumption at age t, preferably maximum age), a model was developed in cooperation with PD Thomas Brey (AWI). The model was built from the following components: Von Bertalanffy growth model, shell size to body mass relation and standard respiration to body mass relation. With these components the growth in body mass per time t and the relation of standard respiration to age could be calculated. The lifetime respiration was then calculated by accumulating respiration, calculated for subsequent small time intervals dt from age zero to the maximum age found in the population of the different species. For a more detailed description of the model see publication I.

- Whole protein content

The evaluation of the protein content in the mantle tissue of ageing bivalves provides information about changes in tissue composition with age. From mantle tissue of differentially aged individuals of the different bivalve species, the whole tissue protein was extracted as described in detail in publication I and III and measured photometrically by the Biuret method (Kresze, 1988).

- Mitochondrial respiration and ROS generation

Mitochondria are supposed to play a key role in the physiological ageing process as they are assumed to be the major source of endogenous oxygen radical generation and are the first to experience oxidative damage by the reactive oxygen species which they produce. Investigation of age-related changes in mitochondrial function will therefore give insight into the physiological ageing of marine bivalves. Isolated mitochondria from the mantle tissue of differentially aged *M. arenaria, L. elliptica* and *A. opercularis* were investigated with respect to age dependent changes of mitochondrial respiration, coupling

of phosphorylation and oxygen consumption (RCR), leak of protons back into the mitochondrial matrix (proton leak), ratio of ADP to oxygen consumption (ADP/O) and the membrane potential at species specific mean environmental temperature i.e. 10°C for *M. arenaria* and *A. opercularis* and 0°C for *L. elliptica* (Publ. II and III). Furthermore, the mitochondrial generation of hydrogen peroxide (H_2O_2) was investigated in different respiratory states.
For *A. colbecki* only preliminary data could be obtained as just a few life animals were available to study mitochondrial functions.

- Mitochondrial enzyme activities

Activities of the key mitochondrial enzymes citrate synthase (CS) and cytochrome c oxidase (COX) were determined at mean annual *in situ* temperature and at a reference temperature of 20°C in mantle tissue of differentially aged individuals of the investigated species. CS is an important mitochondrial matrix enzyme in the citric acid cycle, acting as an acceptor of acetyl-coenzyme A. In contrast COX, is bound to the mitochondrial inner membrane and part of complex IV of the respiratory chain. CS activity was measured after Sidell et al. (1987) and COX activity after Moyes et al. (1997).

Antioxidative defence parameters:

- Superoxide dismutase, catalase, glutathione

The antioxidant capacities of differentially aged individuals of the bivalve species were evaluated by measuring the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase and by the determination of glutathione content in mantle tissue. The glutathione concentration not only yields information about the antioxidant potential of the tissue but also provides information about the redox state of the tissue when both, reduced (GSH) and oxidised (GSSG) glutathione are determined, and the ratio of GSSG/GSH is calculated (Schafer and Buettner, 2001). The antioxidant enzymes SOD and catalase were determined photometrically according to Livingstone et al. (1992) and Aebi et al. (1984) at annual mean temperature of the different species and at 20°C reference temperature. The glutathione content was measured using high performance liquid chromatography (HPLC), the method being modified after Fariss and Reed (1987).

Oxidative stress parameters:

- Lipofuscin

Lipofuscin is often called fluorescent age pigment (FAP) and considered as a physiological proxy of ageing. In several studies its accumulation rate has been found to correlate negatively with longevity (Brunk and Terman, 2002). Lipofuscin is a brown yellow

auto- fluorescent electron dense substance. It represents incompletely degraded damaged cell structures, mainly proteins and lipids (30-70% and 20-50% respectively) and accumulates in the lysosomes, where it is practically indigestible (Terman, 2001; Brunk and Terman, 2002). In the present study, lipofuscin was extracted from the mantle tissue of the bivalves and measured fluorometrically after Vetter et al. (1988).

- Protein carbonyls

Protein modifications induced by reactive oxygen intermediates or by-products of oxidative stress can lead to the formation of carbonyl groups. The presence of carbonyl groups in proteins has therefore been used as a marker of ROS mediated protein oxidation. Consequences of oxidative protein modification can be the loss of enzyme activity and protein function (for review see Berlett and Stadtman, 1997). However, the content of oxidized protein levels does not only reflect the rate of protein oxidation but also the rate of oxidative protein degradation. Oxidized proteins can be degraded by proteasomes and lysosomes, which can be less efficient or abundant with age, increasing the half-life of the oxidized proteins (for review see Dunlop et al., 2002). The carbonyl content in the mantle tissue of the investigated bivalve species was determined photometrically after Levine et al. (1990).

3. Publications

List of publications and declaration of my contribution towards them

Publication I

Eva Philipp, Thomas Brey, Hans-Otto Pörtner, Doris Abele

Chronological and physiological ageing in a polar and a temperate mud clam

Mechanisms of Ageing and Development, in press

I elaborated the concept and initial idea for this paper together with the third and fourth author. I carried out the practical work at the Alfred-Wegener Institute in Bremerhaven and on the Dallmann-Station in Antarctica. Data were analysed by myself and in cooperation with the second author. The first draft of the manuscript was written by myself and revised together with all co-authors.

Publication II

Eva Philipp, Hans-Otto Pörtner, Doris Abele

Mitochondrial ageing in a polar and a temperate mud clam

Mechanisms of Ageing and Development, in press

The scientific ideas for this paper were developed by the second and third author and myself. The laboratory work was conducted and analysed by myself, in cooperation with the third author. I wrote the first manuscript, which was improved in cooperation with the co-authors.

Publication III

Eva Philipp, Thomas Brey, Olaf Heilmayer, Doris Abele, Hans-Otto Pörtner

Physiological ageing in a polar and a temperate swimming scallop

Marine Ecology Progress Series, submitted

I developed the scientific concept of this study in joint cooperation with the fourth and fifth author. Sampling of the polar species was done by S. Gatti and P. Rehm (AWI) in cooperation with the PNRA (Italian National Programme for Antarctic Research). Data were obtained by myself and provided to a minor part by the third author. My manuscript draft was discussed and revised with all co-authors.

Publication I

Chronological and physiological ageing in a polar and a temperate mud clam

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Abstract

We investigated chronological and physiological ageing of two mud clams with regard to the “rate of living theory” (Pearl, 1928) and the “free radical theory of ageing” (Harman, 1956). The Antarctic *Laternula elliptica* (Pholadomyoida*)* and the temperate *Mya arenaria* (Myoida) represent the same ecotype (benthic infaunal filter feeders), but differ in maximum life span, 36 years and 13 years, respectively. *L. elliptica* has a 2-fold lower standard metabolic rate than *M. arenaria*, but its life long energy turnover at maximal age is 3-times higher. When comparing the two species within the lifetime window of *M. arenaria,* antioxidant capacities (glutathione, catalase) are higher and tissue oxidation (ratio of oxidised to reduced glutathione, lipofuscin accumulation) is lower in the polar *L. elliptica* than in the temperate mud clam. Tissue redox state in *L. elliptica* remained stable throughout all ages, whereas it increased dramatically in aged *M. arenaria*. Our results indicate that metabolic rates and maintenance of tissue redox state are major factors determining maximum lifespan in the investigated mud clams.

Keywords

Chronological and physiological ageing; Bivalves; ROS

1. Introduction

In air breathing species, standard metabolic rate (SMR) seems to be a major factor determining maximum life span (MLSP) (Pearl, 1928; Sohal, 1986). According to the “free radical theory of ageing” (Harman, 1956) this is due to the generation of more reactive oxygen species at higher respiration rate, which damage important cell structures like DNA, protein and lipids, thus leading to a faster deterioration of physiological functions and therefore to a reduction of MLSP.

This relationship was intensively investigated in mammals (rodents, humans) and in short lived air-breathing invertebrates (*Drosophila melanogaster, Caenorhabditis elegans*), but to a lesser extent in aquatic ectothermal invertebrates (*Hydra, Mytilus edulis, Margaritifera margaritifera, Sepia officinalis*) (Viarengo et al., 1989; Canesi and Viarengo, 1997; Martinez, 1998; Zielinski and Pörtner, 2000; Ziuganov et al., 2000; Sukhotin and Pörtner, 2001; 2002).

There is sound empirical evidence for an increase in MLSP of aquatic ectotherms from temperate towards permanently cold waters when comparing species of similar lifestyle (Brey, 1991; Brey et al., 1995; Ziuganov et al., 2000; Cailliet et al., 2001; La Mesa and Vacchi, 2001). This extension of the chronological life span in ectotherms living at permanent cold temperatures requires a delay in physiological ageing. Here both, the “rate of living theory” (Pearl, 1928; Sohal, 1986) and the “free radical theory of ageing” (Harman, 1956) present a convincing yet unconfirmed explanation: At lower temperatures, the lower metabolic rates and correspondingly lower reactive oxygen species propagation from aerobic mitochondrial activity result in a slower rate of physiological ageing. Hence, temperature affects the relationship between chronological age (i.e. absolute life time) and physiological age (i.e. a less clearly defined measure of physiological fitness), which are positively correlated, but not necessarily linearly and in the same way in each species.

In this study, we compare chronological and physiological ageing of two mud clams, the Antarctic *Laternula elliptica* (Pholadomyoida) and the North Sea *Mya arenaria* (Myoida), in order to understand why the polar clam reaches 3-times the maximum chronological age of the temperate species (±36 y vs. ±13 y). MLSP of *M. arenaria* is estimated to be 10-12 years on the Atlantic east coast (Strasser, 1999) which is in line with the MLSP of 13 years found in our samples. The MLSP of *L. elliptica* is based on over 650 individual age determinations (this study and T. Brey pers. comm.) with only 2 animals reaching 36 years. Both species are of the same ecotype (deep burrowing filter feeding clams) and represent important key species in their respective habitat, but are evolutionary adapted to different temperature regimes. Antarctic waters are characterized by year round stable low temperatures between -1.9 and +2°C (Clarke, 1991). In contrast, species from temperate

coastal waters can experience pronounced temperature fluctuations between 0 and 18°C and even higher. Most Antarctic marine ectotherms are well adapted to these particular polar conditions, showing a narrow window of thermal tolerance as compared to temperate relatives. Regarding individual growth, reproduction and metabolism, they live their life in the slow lane (Clarke, 1983; Clarke, 1988).

Although *L. elliptica* and *M. arenaria* belong to different bivalve subclasses, the similarity in lifestyle in different environments appears just as crucial for such comparisons as a close phylogenetic relationship. Moreover longer MLSPs in Antarctic animals have also been reported in a comparison of more closely related species, *Adamussium colbecki* and *Aequipecten opercularis* (Heilmayer et al., 2003; Heilmayer et al., 2004). *Mya arenaria* is known from America since the Pliocene. In Europe it became extinct at the beginning of the Pleistocene and is thought to have been reintroduced either in the 13th or in the 16th or 17th century (Petersen et al., 1992). *Laternula elliptica* has a continuous history in Antarctic waters, with late Pliocene records from the Northern Antarctic Peninsula (Soot-Ryen, 1952; Jonkers, 1999).

We analysed changes of oxidative stress levels with age and their potential impact on physiological functions in the two mud clam species based on 3 types of physiological ageing parameters: (i) basic metabolic parameters (standard metabolic rate, tissue protein content), (ii) oxidative defence parameters (superoxide dismutase, catalase, glutathione) and (iii) markers for oxidative damage (lipofuscin, protein-carbonyls).

2. Material and Methods

2.1. Sampling and maintenance

2.1.1. *Laternula elliptica*

Antarctic *Laternula elliptica* were collected by scuba divers in Potter Cove, King George Island, South Shetland Islands (62°14`S, 58°40`W) in November-February 2002/2003 in 5 to 10 m water depth. Temperature and salinity at 10 m were about -1 to +2°C and 34 PSU. Animals were kept in aquaria with natural seawater from the cove at 0°C in a constant temperature room (CT room) at the Argentinean base Jubany (Dallmann-Laboratory) for several days prior to experiments and analyses. Water was exchanged once a week. Measurements of metabolic rate and enzymes activities as well as lipofuscin measurements were carried out at the Dallmann-Laboratory. For all other analyses, samples were freeze clamped immediately after sacrificing of the animals and stored in liquid nitrogen for deep frozen transportation to the Alfred Wegener Institute, Bremerhaven.

2.1.2. *Mya arenaria*

North Sea *Mya arenaria* were sampled with a box corer at a shallow subtidal muddy site close to Harlingen (53°09`N, 05°19 ´E), The Netherlands. Animals were gently cleaned from sediment and maintained in thermoboxes with natural seawater during transportation to Bremerhaven. At the Alfred Wegener Institute, animals were transferred to seawater (10°C, 28 PSU) aquaria with a 20 cm sediment layer. On contact with the sediment the animals immediately started burrowing. Animals were fed plankton tablets (REWE, Germany) twice a week, and individuals carried plankton material in the stomach when dissected.

2.2. Age determination

Age of individual *Mya arenaria* was inferred from shell length and a Von Bertalanffy growth model (VBGM) based on length-at-age data of the same subtidal population:

$$S_t = 117.17 * (1-e^{-0.12 * (t+0.054)}) \qquad \text{(N = 181 data points from 23 animals)}$$

The length at-age-data were obtained by isotope analysis (Krantz et al., 1984; Wefer and Berger, 1991) and the investigation of shell growth bands (MacDonald and Thomas, 1980; Brousseau and Baglivo, 1987) of *M. arenaria* individuals. For isotope analysis, calcium carbonate powder was sampled from the outer shell layer of four individuals in equally spaced (~1 mm) dorso-ventral series using a small dental drill (bit size 0.5 mm). $\delta^{13}C$ and $\delta^{18}O$ values were determined in the Stable Isotope Laboratory of the Institute of Palaeontology of the University of Munich with a coupled analysing system Gasbench II / Delta *plus* (Thermo Finnigan). Isotope profiles ($^{13}C/^{12}C$) and ($^{18}O/^{16}O$) were compared with corresponding growth checks visible on the outer shell surface and in x-ray photographs, to distinguish between age rings and disturbance rings as described in Heilmayer et al. (2003; 2004). In these four *M. arenaria* individuals and 19 additional shells, where growth bands were clearly visible on the outer shell and x-ray, the distance of identified year rings to the umbo was then determined resulting in 181 width-to-age data points which were transformed to length-to-age data points by a width – length conversion factor of 0.617 inferred from 259 *M. arenaria* shells. A length-to-age curve was then fitted using a Von Bertalanffy growth model (VBGM) (Fig.1).

In contrast to *M. arenaria*, the relation between shell length and age was found to be more variable in *Laternula elliptica*. Hence, every individual was aged directly by counting the annually formed growth checks (Brey and Mackensen, 1997) in polished cuts through the shell umbo. A few damaged shells could not be aged as previously described. For these individuals age was inferred from a length-at-age table (age “y” found at shell length “x”), based on the 347 directly aged shells.

2.3. Metabolic rate

Prior to respiration measurements, *M. arenaria* and *L. elliptica* were maintained without food for two and three days, respectively, in order to eliminate impact of specific dynamic action (SDA) on respiration.

Standard metabolism was approximated by standard respiration measured in a multi-channel modified intermittent flow system and oxygen microoptodes connected to a Microx TX 2–array (® PreSens GmbH), as previously described in Gatti et al. (2002) and Heilmayer and Brey (2003). The respiration chambers were perspex cylinders of different volumes, adjustable to the size of the animals (150-990 ml). Experimental conditions were maintained at 10°C and 27 PSU in *M. arenaria*, and at 0 ± 0.4°C and 34 PSU in *L. elliptica*. Microoptodes were calibrated to 100% oxygen solubility in air-saturated and to 0% in N_2-saturated seawater at both experimental temperatures.

Bivalves were allowed to accommodate to the respiration chambers over night. Only actively respiring animals that had their siphons extended into the surrounding water were measured. A measurement cycle was initiated by switching the system from “flow-through” to “closed-circle flow” status, starting the first measurement run. The water in the closed system was continuously circulated by a peristaltic pump (Spetec Perimax 127) and the decrease in oxygen content from 100% to about 70% oxygen over time was recorded. At about 70% oxygen, the system was switched back to “flow-through” and flushed with water to achieve 100% oxygen saturation, then a new run started. Two to three runs, taking about 4 to 5 h each, were carried out per animal. Immediately upon completion of the measurement cycle, the animal was dissected and soft tissue wet mass was determined. The soft tissue was dried at 60°C for at least 3 days to determine dry mass. Tissues were combusted at 500°C for 5 h (Ahn and Shim, 1998) and ash mass was subtracted from dry mass to obtain ash free dry mass.

Standard metabolic rates (SMR) were determined after subtraction of the microbial oxygen demand, determined in a parallel blank chamber. Percent O_2 saturation was transformed to micromoles of dissolved oxygen in seawater using known values of oxygen solubility (Benson and Krause, 1984; Heilmayer and Brey, 2003) and converted to µg O_2 by 1µmol O_2 = 32.62µg O_2.

Life-long aerobic expenses were approximated using lifetime respiration, i.e. total aerobic energy gain LR_t of an individual until age t. A model to estimate LR_t was build from the following components:

The Von Bertalanffy growth model (Von Bertalanffy, 1934) describes the relation between body size S_t and individual age t:

$$S_t = S_\infty * (1 - e^{-K * (t - t_o)}) \quad (1)$$

where S_∞ is asymptotic maximal size, K is the acceleration constant, t is age, and t_o is age at which length would be zero. With the exponential body size to body mass relation:

$$M_t = a1 * S_t^{b1} \quad (2)$$

growth in size is transformed into growth in body mass:

$$M_t = a1 * S_\infty^{b1} * (1 - e^{-K * (t - t_o)})^{b1}$$

$$\Leftrightarrow M_t = M_\infty * (1 - e^{-K * (t - t_o)})^{b1} \quad (3)$$

where M_∞ is asymptotic body mass. Standard respiration R_t is related to body mass M_t by the exponential relation

$$R_t = a2 * M_t^{b2} \quad (4)$$

and hence to age t by

$$R_t = a2 * (M_\infty * (1 - e^{-K * (t - t_o)})^{b1})^{b2}$$

$$\Leftrightarrow R_t = a2 * (a1 * S_\infty^{b1} * (1 - e^{-K * (t - t_o)})^{b1})^{b2} \quad (5)$$

Lifetime respiration LR_t is computed by numerical integration, i.e. by accumulating respiration computed for subsequent small time intervals dt from age zero to age t:

$$LR_t = \Sigma (R_t * dt) \quad (6)$$

Additional respiration data for *L. elliptica* obtained by Ahn and Shim (1998) were included in the analysis. The specific parameters used for calculating lifetime respiration are shown in table 1.

2.4. Enzyme assays

For enzyme assays, animals were dissected and mantle tissue freeze clamped and stored in liquid nitrogen. Assay temperature was 0°C for *L. elliptica*, 10°C for *M. arenaria*, and 20°C for reference. All assays were measured spectrophotometrically (Pharmacia, Shimatzu UV 1202 for *L. elliptica* and Beckman DU 7400i for *M. arenaria*). Data are expressed as international units (µmol of substrate converted to product min^{-1}) per mg protein. Protein content of the extracts was determined by the Biuret method (Kresze, 1988).

2.4.1. Superoxide dismutase

Superoxide dismutase (SOD) was determined after Livingstone et al. (1992). Frozen mantle tissue was ground in liquid nitrogen and homogenised with a micropistill in Tris buffer (20 mM TRIS-HCl, 1 mM EDTA, pH 7.6) 1:8 (w/v). Samples were centrifuged for 3 min at 14000 rpm and at 2°C. SOD activity was measured as degree of inhibition of the reduction of cytochrome c by superoxides generated by a xanthine oxidase/xanthine system at 550 nm in 43 mM potassium buffer with 0.1 mM EDTA, pH 7.8. 1 Unit SOD causes a 50% inhibition under the assay conditions.

2.4.2. Catalase

Catalase activity was determined after Aebi et al. (1984). Frozen mantle tissue was ground in liquid nitrogen and homogenised with a micropistill in 50 mM phosphate buffer (50 mM KH_2PO_4, 50 mM Na_2HPO_4, pH 7.0) with 0.1% Triton x-100 at 1:5 (w/v). Samples were centrifuged at 13000 g for 15 min at 2°C. The activity was determined by recording the time of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.4 at 240 nm (1 unit).

2.5. Glutathione content

The concentrations of the oxidised (GSSG) and the reduced form of glutathione (GSH) were measured after Fariss and Reed (1987) using high performance liquid chromatography (HPLC). The principle of the measurement is the derivatisation of the thiols with dinitrofluobenzene (DNFB). GSH oxidation during extraction is prevented by iodoacetic acid (IAA) binding of GSH. Immediately after dissecting an animal, the tissue was freeze clamped to prevent GSH oxidation and kept in liquid nitrogen. Tissues were ground in liquid nitrogen and homogenised with ice-cold perchloric acid (PCA) (10% with 2 mM Bathophenanthrolinedisulfonic acid) at 1/10 (w/v). Following centrifugation at 15000 g and 4°C for 5 min, 500 µl of the supernatant was transferred to a fresh reaction vial and 10µl of the pH indicator (1 mM m-cresol purple in H_2O with 0.5 M iodoacetic acid) and 50µl internal standard (1 mM gamma-glutamyl-glutamat in 0.3% PCA) added. Samples were adjusted to pH 8.5 with 4 M KOH with 0.3 M n-Morpholinopropanesulfonic acid and incubated for 45 min at room temperature, to allow iodoacetic acid to bind the GSH. After 5 min centrifugation at 15000 g and 4°C, 1% DNFB (1% 1-fluoro-2.4-dinitrobenzene in ethanol) was added to the supernatant at a 1:3 ratio and incubated in a dark vial for 24h at room temperature without shaking. Thereafter samples could be kept frozen for at least 6 months until analysis.

Prior to injection into the HPLC, thawed samples were again centrifuged for 1 min at 7500g and 4°C, to remove remaining PCA and the supernatant filtered through a 0.2 µm

nylon membrane filter. Samples were transferred to dark autosampler vials and injected using an autosampler, thermostatted to 4°C. Separation was achieved on a NH_2-spherisorb column (240x4mm, 5µm particles) at 39°C using a binary solvent system of A: 80% methanol/water and B: 80% solvent A and 20% acetate stock (272g Na-acetate-trihydrate diluted in 122 ml water plus 378 ml glacial acetic acid). Both solvents were degassed and filtered (0.45 µm pore size) prior to use. Flow rate was 1.2 ml min^{-1} at a maximal backpressure of 2500 psi. The gradient program was: 90% A/10% B for 12 min, followed by 30 min of linear gradient elution to 45% A/55%B and a subsequent 8 min hold. Thereafter the system was returned to the initial conditions within 5 min and re-equilibrated for 15 min.

2.6. Proteinoxidation

Detection of protein carbonyl groups as a measure of protein oxidative modifications was carried out after Levine et al. (1990). Carbonyls react with the carbonyl-specific reagent 2.4-dinitrophenylhydrazine (DNTP) and can be measured spectrophotometrically at 360 nm (molar extinction coefficient E = 22000 M^{-1} cm^{-1}).

Samples were homogenised in 5 ml of 50 mM HEPES containing 125 mM KCl and protease inhibitors (0.5 mg/ml leupeptine, 0.7µg/ml pepstatine, 40µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinine, 1.1 mM EDTA, 0.6 mM $MgSO_4$) and centrifuged at 31000 g for 15 min. 0.4 ml of supernatants were incubated at room temperature for 1h with 1.4 ml 10 mM DNTP in 2 M HCl. Absorbance blanks of samples were run without DNTP. During the incubation, samples were vortexed every 15 min. After 1 h, 0.2 ml of 100% TCA were added to precipitate the protein and centrifuged for 10 min at 11000g. The protein pellet was washed 3 times with 1 ml ethanol: ethylacetate (1:1), resuspended in 0.6 ml 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH = 2.3) and incubated in a water bath for 30 min at 37°C until complete resuspension. Samples were kept in the refrigerator over night and measured the following day in the photometer (Pharmacia Biochrom 4060) with guanidine hydrochloride as a reference. The protein content of the samples was determined by the Bradford method using bovine serum albumin as a standard.

2.7. Fluorescent age pigment, lipofuscin

Lipofuscin contents were determined by an extraction method modified after Vernet et al. (1988). Frozen mantle tissue was ground in liquid nitrogen and homogenised (1/20 w/v) in a chloroform-methanol solution (2:1 v/v). The homogenate was mixed with 100 mM $MgCl_2$ (1 ml per each 4 ml of Chloroform/methanol) solution. After 15 min centrifugation at 2000 g the chloroform phase was collected and mixed with distilled water (1 ml per 4 ml initial

chloroform/methanol). After 20 min at 2000 g and at 0°C, the chloroform phase was again collected and could be measured in the fluorometer. An emission spectrum was obtained at an excitation wavelength of 350 nm. The fluorescence intensity of each sample was determined at an emission maximum of 415 nm for *L. elliptica* and 464 nm for *M. arenaria*. According to Hill and Womersley (1991), lipofuscin concentrations were expressed as relative fluorescent intensity (RFI) using 0.1 µg quinine sulphate per ml 1 N H_2SO_4 as standard.

2.8. Protein content

Mantle tissue of both species was lyophilised and then reduced to small pieces with a spatula. 3% NaOH was added at a 1:50 (w/v) ratio and shaken for 20 h at room temperature to hydrolyse the protein. Subsequently, samples were centrifuged for 20 min at 5000 rpm to remove cell debris and cooled on ice to facilitate protein precipitation. A two-fold volume of ice-cold 60% TCA was added (30 ml per initial g tissue dry mass) to precipitate the protein. After 20 min at 5000 rpm, the supernatant was discharged and the pellet dissolved in 3% NaOH using 20 ml per initial g tissue dry mass. Protein concentration was determined spectrophotometrically with the Biuret method.

2.9. Statistical analysis

Relationships between parameters and age and differences between species were evaluated by analysis of variance (ANOVA) and analysis of covariance (ANCOVA) after testing for normality by the Kolmogorov-Smirnov test.

3. Results

3.1. Whole animal metabolism and tissue protein content

Whole animal respiration rates, measured at *in situ* temperature (*M. arenaria* 10°C, *L. elliptica* 0°C) were about 2-fold higher in *M. arenaria* compared to *L. elliptica* (Fig. 2) at the same AFDM. The lifetime aerobic expenses *of M. arenaria* and *L. elliptica* computed from the corresponding models relating body size to age, body mass to body size and respiration rate to body mass are shown in Fig. 3. Life-long aerobic expenses, accumulated at maximum age, are 3-times higher in *L. elliptica* ($8.98 * 10^5$ J ind^{-1} at 36 years of age) than in *M. arenaria* ($3.00 * 10^5$ J ind^{-1} at 13 years of age).

Measurements of tissue protein content in the mantle tissue revealed an increase in tissue protein content per g dry mass with age in *L. elliptica*, but not in *M. arenaria* (Fig. 4). In *L. elliptica*, protein concentrations decreased significantly from around 360 mg to 200 mg

protein g^{-1} dry mass between 4 and 36 years of age. In *M. arenaria* values varied around 450 mg protein g^{-1} dry mass throughout all age classes.

3.2. Age dependent changes of antioxidant defence mechanisms

The activity of the antioxidant enzymes superoxide-dismutase (SOD) and catalase (CAT) were constant throughout all ages in both species. A between species comparison yielded similar SOD activities in both animals, independently of whether assays were run at 20°C reference temperature or at *in situ* temperature (10°C for *M. arenaria* and 0°C for *L. elliptica*) (Fig. 5, A). In contrast, catalase (CAT) activities were distinctly higher in *L. elliptica* than in *M. arenaria* at 20°C assay temperature. At *in situ* temperature (10°C) catalase activity was below detection limits in *M. arenaria* mantle (Fig 5, B). Q_{10} values were close to 1 for SOD in both species and were 1.8 for CAT in *L. elliptica*. Due to the low CAT activity at 10°C, Q_{10} for CAT of *M. arenaria* could not be calculated.

Throughout the lifetime of *M. arenaria*, the concentration of total tissue glutathione (tGSH: 2[GSSG] + [GSH]) was higher in *L. elliptica* than *M. arenaria*, (Fig. 6A). Old *L. elliptica* specimens (>15 years) showed tGSH values in the same range as old *M. arenaria*. tGSH concentrations in *L. elliptica* declined significantly with age (Fig. 6A) from approximately 850 to 380 nmol tGSH g^{-1} wet mass, whereas in *M. arenaria* mantle tissue tGSH values increased significantly with age from approx. 200 to 420 nmol tGSH g^{-1} wet mass (Fig. 6A). Mantle tissue of *M. arenaria* was more oxidized (ratio GSSG:GSSG) than *L. elliptica* throughout all ages, and tissue oxidation increased significantly with age in *M. arenaria*, due to an increase in GSSG (Fig. 6B), whereas in *L. elliptica* age-dependent tissue oxidation remained constant (Fig. 6C).

3.3. Age-dependent changes in oxidative damage parameters

The deposition of both lipofuscin and protein carbonyls in mantle tissue of *L. elliptica* (Fig. 7, 8) increased significantly with age. In contrast to the findings in *L. elliptica*, the carbonyl content decreased with age in *M. arenaria* (Fig. 8). Lipofuscin contents also decreased during the first 5 years of life but increased thereafter (Fig. 7). Within the lifetime window of *M. arenaria* lipofuscin content was generally higher in *M. arenaria* compared to *L. elliptica*. Protein carbonyl contents were in the same range in young specimens from both species. Given the pronounced increase in protein carbonyl content with age in *L. elliptica* as opposed to the age dependent decline in *M. arenaria*, protein carbonyl content was higher in aged *L. elliptica* mantle tissue, compared to aged *M. arenaria*.

4. Discussion

Does MLSP rely more on standard metabolic rate or more on lifetime respiration?

The species with the distinctly shorter life span, *M. arenaria*, shows a 2-fold higher standard metabolic rate (SMR) at *in situ* temperature (Fig. 2), but 3-times lower life-long aerobic expenses (Fig. 3) than the longer-lived *L. elliptica*.

This indicates that MLSP relates more to the instantaneous rate of respiration than to cumulative lifetime respiration. Our findings coincide with those obtained in a study of two rodent species with different MLSP, where the metabolic potential (O_2 consumed/g during the whole life span= MSLP x SMR) was determined, a parameter comparable to the lifetime aerobic expenses calculated in the present study (Sohal et al., 1993). The longer lived *Peromyscus leucopus* showed a higher metabolic potential than the shorter lived *Mus musculus,* albeit both species showed similar SMRs. This indicates that other factors besides SMR crucially affect MLSP, e.g. antioxidant capacities.

Do antioxidant defence capacities affect MLSP?

In the present study the long lived *L. elliptica* showed generally higher antioxidative capacities (catalase, glutathione) than the short lived *M arenaria*. This is in line with a study by Estevez et al. (2002), who found higher tocopherol and carotene contents in the digestive gland of *L. elliptica* compared to *M. arenaria*. Sohal et al. (1993) also found higher antioxidant capacities in the longer lived rodent species, together with lower rates of mitochondrial ROS generation, which resulted in lower oxidative damage accumulation and were held responsible for the longer MLSP. In short-lived cephalopods with typically high standard metabolic rates, Zielinski and Pörtner (2000) found low levels of antioxidative defence which relate to the short MLSP of this group.

Distinct but opposite trends in glutathione concentration were found with age in *L. elliptica* and *M. arenaria* (Fig. 6). In *L. elliptica* glutathione decreased significantly with age, which is in line with several findings in humans, rats, insects and a marine bivalve (Sohal et al., 1987; Sanz et al., 1996; Canesi and Viarengo, 1997; Hernanz et al., 2000). Interestingly, despite lower glutathione concentrations at old age, the GSSG:GSH ratio remained constant throughout all ages in *L. elliptica*, reflecting unchanged tissue oxidation state. The clam might adjust the glutathione content to an age-related decrease in SMR, as it seems sufficient to keep the tissue oxidation stable in aged animals. A decrease in SMR with age was found in the mussel *Mytilus edulis* (Sukhotin and Pörtner, 2001), but our data on *L. elliptica* did not allow for the analysis of this effect. It is unclear why tGSH concentration decreases, whereas catalase activity remains stable with age in *L. elliptica* as both destroy H_2O_2. The universal presence of glutathione in the mitochondrial matrix

and different affinities of the involved enzymes, catalase (high Km) and glutathione peroxidase (low Km), for H_2O_2, may be responsible for the patterns observed (Chance et al., 1979; Phung et al., 1994; Schafer and Buettner, 2001; Rebrin et al., 2003; Barja, 2004). In *M. arenaria* tGSH and the state of tissue oxidation increased significantly with age, indicating increased ROS scavenging in older animals. Leeuwenburgh et al. (1994) also reported an increase of tGSH with age in rat skeletal muscle along with increased levels of lipid oxidative damage markers, but tissue oxidation decreased in the rat skeletal muscle with age. Altogether it appears that the conservation of tissue redox state might be crucial to control the physiological ageing process which determines MLSP. A low GSSG:GSH ratio is important to ensure the availability of GSH in situations of oxidative stress and, moreover, GSSG accumulation stimulates protein S-glutathiolation that can cause irreversible loss of protein function (Klatt and Lamas, 2000).

In contrast to glutathione, antioxidant enzyme activities in *L. elliptica* and *M. arenaria* were independent of age which implies a continuous need for a stable antioxidative protection system until old age. Data on age-related changes in antioxidative enzyme activities in other marine ectotherms (fish, crustaceans, worms, bivalves, cephalopods), however, show no conclusive pattern (Viarengo et al., 1989; Viarengo et al., 1991; Buchner et al., 1996; Mourente and Diaz-Salvago, 1999; Zielinski and Pörtner, 2000; Sukhotin et al., 2002; Correia et al., 2003; Passi et al., 2004). A thorough analysis of the response of enzymatic antioxidants to changes in physiological functions with age (metabolic rates, animal activity, body composition) may lead to a more detailed understanding of the role of various antioxidants in determining MLSP in aquatic ectotherms of different lifestyles and temperature regimes.

Lipofuscin content increased continuously with age in *L. elliptica,* as found in several other species (Leeuwenburgh et al., 1994; Zielinski and Pörtner, 2000; Bluhm et al., 2001; Sukhotin et al., 2002). A high share of unsaturated fatty acids in the membranes, a pattern frequently detected in low temperature fish, could facilitate lipid peroxidation, as they are more vulnerable to oxidative damage (Cossins et al., 1978; White and Somero, 1982). In *L. elliptica*, however, the level of total fatty acid unsaturation in soft tissue is similar to warm water bivalve species (Ahn et al., 2000). The comparison of two scallops, the Antarctic *Adamussium colbecki* and the temperate *Pecten jacobaeus*, resulted in a similar picture: Viarengo et al. (1995) found similar levels of unsaturated fatty acids, but distinctly higher levels of branched and short-chain saturated fatty acids in membranes of the digestive gland of *A. colbecki*. A high amount of unsaturated fatty acids would accelerate lipid peroxidation and therefore be adverse to a long MLSP, as it was found in air breathing mammals and birds, where higher unsaturated fatty acid contents in whole

tissues and mitochondria are correlated with shorter life spans (Javouhey-Donzel et al., 1993; Pamplona et al., 1998; Herrero et al., 2001). The maintenance of low levels of unsaturated fatty acids as seen in *L. elliptica* might represent an antioxidant, presumably life-prolonging strategy. As age could be determined individually in *L. elliptica*, the relationship of physiological ageing parameters with age, and respectively with body mass, was explored (Table 2). P values were highly significant for all parameters, except the GSSG:GSH ratio, irrespectively of whether plotting against age or mass, whereas F values were always slightly higher for the parameter/age than the parameter/mass relationship, especially in the case of lipofuscin. This indicates a closer dependency of lipofuscin accumulation on age than on mass. In *M. arenaria* a distinction between age and body mass was unattainable as individual ages were calculated using a VBGM.

In contrast to findings in *L. elliptica*, lipofuscin content decreased in mantle tissue of *M. arenaria* with age during the first 5 years and increased thereafter (Fig. 7). A decrease from relatively high initial lipofuscin concentrations during early lifetime can be interpreted as a process of “rejuvenation” (Terman, 2001), associated with the dilution of waste material during intensive growth of the young. Such rejuvenation has also been found in *Mytilus edulis* (Hole et al., 1995). The imbalance between higher metabolic rates of young *M. arenaria* causing high generation rates of reactive oxygen species and the constant antioxidant defence might cause the high lipofuscin content in young *M. arenaria*. Conversely in specimens 5 years and older, a lower damage dilution due to slower growth and cell division may enhance lipofuscin particle density despite a decrease in SMR.

If not with unsaturated fatty acid levels, the increase in lipofuscin with age in *L. elliptica* may be related to soft tissue iron content. Iron is a transition metal, which propagates lipid radical formation. Estevez et al. (2002) found higher lipid radical formation in digestive gland homogenates of *L. elliptica* compared to *M. arenaria* going along with higher iron content in its tissues. Assuming that iron accumulates with age in *L. elliptica* soft body, this could enhance lipid radical formation and contribute to lipofuscin accumulation in older animals, as observed in human glia cells, cultured rat heart myocytes and marine ascomycetes (Thaw et al., 1984; Totaro et al., 1986; Marzabadi et al., 1988). Additionally, low temperature induces slow cell turnover rates, as documented for cold stenothermal Antarctic fishes compared to a eurythermal species (Brodeur et al., 2003). This may lead to sustained accumulation of oxidative damage products in the Antarctic *L. elliptica* (Hirsch, 1978; Brunk and Terman, 2002).

Nevertheless, absolute lipofuscin values at the same chronological age were always higher in *M. arenaria* compared to *L. elliptica*. In line with the more oxidized redox state

(GSSG:GSH) this argues for age independent higher oxidative stress levels in *M. arenaria*, which may contribute to a reduced MLSP.

The marker for protein oxidation, protein carbonyl content, decreased significantly in *M. arenaria* but increased in *L. elliptica* with age (Fig. 8). As demonstrated in human fibroblasts (Sitte et al., 1998) protein carbonyls, in contrast to lipofuscin, can be readily degraded and thus effectively removed from the cells. Fast cell division, increasing tGSH content and continuously high protein degradation rates might prevent or even decrease carbonyl accumulation in *M. arenaria* with age. However, when excluding less than 4 year old bivalves from the analysis, the significant decrease in carbonyl content with age was abolished. Again, a slow down in growth and cell division in aging animals might lead to stable protein carbonyl contents in older individuals. Arbitrary grouping of the bivalves in different age groups is, however, statistically incorrect, and the above assumption should therefore be handled with care. Nevertheless, a slow down of protein carbonyl degradation or dilution with age would be in line with the enhanced lipofuscin accumulation found in *M. arenaria* individuals above 5 years of age.

In *L. elliptica* the protein carbonyl content increased with age, which is in line with several vertebrate and invertebrate studies, reviewed by Sohal (2002). As tissue redox state (GSSG:GSH) remained constant with age in *L. elliptica*, the increase in carbonyl content seems to reflect progressive accumulation of oxidised proteins rather than an increase in protein oxidation rate with age. In fish and invertebrates *in vivo* protein synthesis rates are lower in Antarctic compared to temperate species at environmental temperature (Storch et al., 2003). Lower protein synthesis rates in *L. elliptica* compared to *M. arenaria* may therefore result in slower protein turnover leading to increased presence of more severely oxidised proteins. As extensively oxidised proteins become more resistant to proteolysis (Sitte et al., 1998; Grune et al., 2004) this may lead to an accumulation of protein carbonyls with age, which may further be enhanced by a decrease of proteolytic activity with age, as shown in mammalian studies (Starke-Reed and Oliver, 1989; Grune et al., 2001). Additionally, according to Ahn et al. (2003), muscle proteins may be important energy reserves in *L. elliptica*, which enable the animals to survive prolonged periods of food-limitation in Antarctic waters. Consequently, a pronounced degradation of oxidised proteins might be avoided and protein oxidation tolerated in *L. elliptica*, to preserve protein energy stores. Additionally, lipofuscin accumulation in aged *L. elliptica* may exacerbate the impairment of protein degradation and storage of oxidized tissue protein, as shown by Sitte et al. (2000) for human fibroblasts cultures.

Along with the age-dependent increase in protein carbonyls in *L. elliptica* tissue, a decrease in protein content of mantle dry mass was found, whereas in *M. arenaria* the

carbonyl content decreased and the protein content per mantle dry mass remained stable (Fig. 4). Age related loss of muscle proteins is well documented in humans and rats (Holloszy et al., 1991; Booth et al., 1994), and is presumably due to declining protein synthesis with age as shown for mitochondrial and mixed muscle protein in vertebrates (human, mouse) and invertebrates (*Drosophila*) (Marcus et al., 1982; Bailey and Webster, 1984; Welle et al., 1993; Rooyackers et al., 1996; Yarasheski, 2003). Declining protein synthesis rates with age and the use of proteins as energy source in *L. elliptica* might result in an imbalance of protein synthesis and protein usage in aged individuals, leading to a decrease in protein content with age. This may become problematic during prolonged periods of food limitation, in which survival of aged *L. elliptica* individuals may be hampered by the lack of energy reserves.

It remains unclear, to what extent and at which concentration lipofuscin and protein carbonyls impair cell functions. Terman (2001) proposed a lower degradation capacity of lipofuscin-loaded lysosomes, leading to an accumulation of damaged cell constituents, including mitochondria, within the cell. This may exacerbate oxidative stress and compromise physiological function, including mitochondrial energy production. On the one hand our study does not confirm the “garbage theory of ageing” (Terman, 2001) as despite significant accumulation of lipofuscin and protein carbonyls with age, *L. elliptica* keeps tissue redox state in balance and grows 3-times older than *M. arenaria*. The *M. arenaria* data, on the other hand, corroborate this hypothesis by showing generally higher levels of lipofuscin contents and tissue oxidation at all ages compared to *L. elliptica*, which could indicate more damaged and therefore ROS generating mitochondria in *M. arenaria* tissue. Further studies are required to understand the influence of oxidative damage on the change in physiological functions with age in marine mud clams.

Conclusions

The long-lived polar *L. elliptica* showed lower SMR than the shorter lived temperate *M. arenaria*, but higher lifetime aerobic turnover at maximum age. This may indicate that the MLSP may depend more on the average rate of respiration than on the magnitude of lifetime aerobic energy turnover. Furthermore, higher antioxidant capacities and a more reduced tissue oxidation state throughout life seem to lead to the observed higher MLSP in the polar mud clam. An increase of oxidative damage parameters with age in *L. elliptica* does obviously not accelerate the physiological ageing process, as the species eventually lives three times longer than *M. arenaria*. The lower metabolic rates and maintenance of a low tissue redox state found in *L. elliptica* compared to *M. arenaria* may enable the polar species to reach a higher MLSP. We are currently undertaking measurements of the

changes of mitochondrial functions with age in both species, to further elucidate the different ageing patterns found in this study.

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Tables:

Table 1: Equations used for lifetime respiration model of *M. arenaria* and *L. elliptica.*

	M. arenaria	*L. elliptica*
Growth (VBGM) [mm, years]	$S_t = 117.17 * (1-e^{-0.12 * (t+0.054)})$ N = 181, $r^2 = 0.934$	$S_t = 101.39 * (1-e^{-0.060 * (t + 0.00)})$ N = 347, $r^2 = 0.806$
Size-mass [mm, mgAFDM]	logM = 3.029 * logS-2.623 N = 90, $r^2 = 0.898$, p<0.001	logM = 3.074*logS-2.531 N = 28, $r^2 = 0.947$, p<0.001
Respiration – Mass [J/d, J]	logR = 0.86*logM-1.833 N = 19, $r^2 = 0.70$, p<0.001	[a] logR = 0.888*logM - 2.067 N = 74, $r^2 = 0.777$, p<0.001

a) Respiration data from the present study and Ahn and Shim (1998).

Table 2: *Laternula elliptica*: Effects of age and body mass on physiological ageing parameters analysed with linear regression (ANOVA).

Physiological Ageing Parameter	**vs. Age** F	**P**	**vs. Body Mass** F	**P**	**N**	**Age Range (y)**	**Mass Range (mg AFDM)**
protein content	35.5	<0.001	25.76	<0.001	31	4-36	433-3062
tGSH	54.05	<0.001	40.15	<0.001	29	2-22	100-2563
GSSG	22.91	<0.001	18.59	0.002	28	2-22	100-2563
GSSG: GSH	3.334	0.0809	2.677	0.1158	25	2-22	100-2563
lipofuscin	95.28	<0.001	41.42	<0.001	23	2.5-36	135-3279
protein carbonyls	21.64	<0.001	17.25	<0.001	28	4-28	227-3506

Figures:

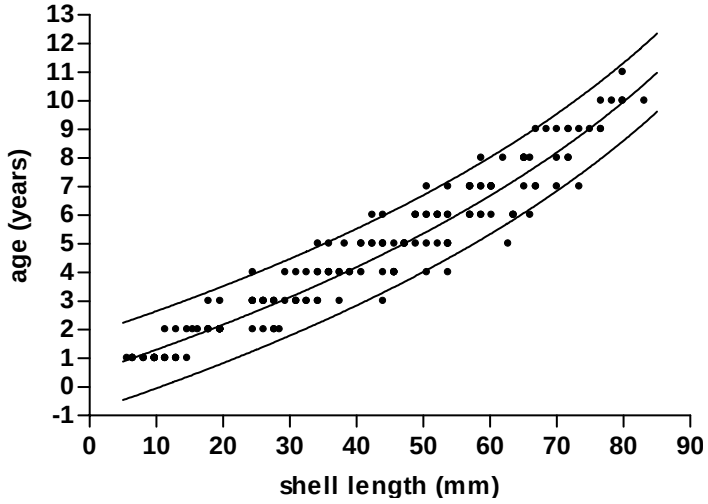

Fig.1: Inverse Von Bertallanffy growth model (VBGM) of *M. arenaria* with the upper and lower 95% confidence limits, inferred from 181 size-at-age data points (23 individuals, age range = 1-10 years).

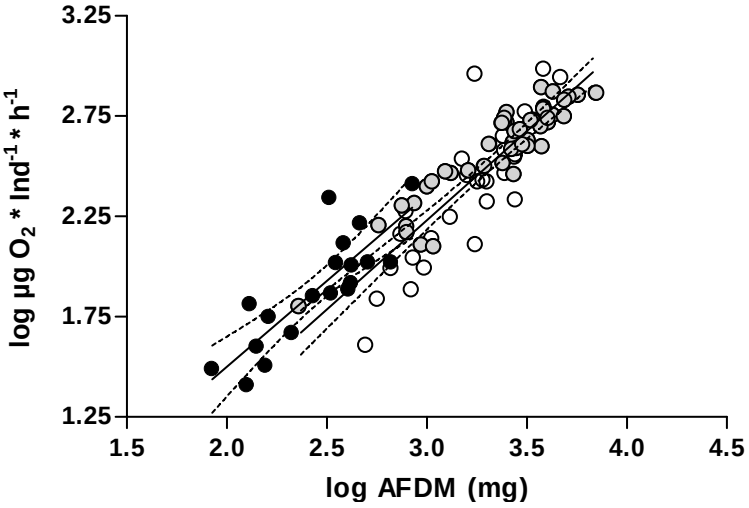

Fig. 2: Respiration rates (μgO_2 Ind h) of *M. arenaria* (filled circles, N = 19, mass range = 85-852 mg AFDM) and *L. elliptica* (open circles: this study, N = 28; grey dots: data from Ahn and Shim (1998), N = 45, mass range = 85-852 mg AFDM) measured at *in situ* temperatures (10°C *M. arenaria*, 0°C (0.8°C Ahn and Shim) *L. elliptica*). *M .arenaria*: log R = 0.86 * log (mg AFDM) -0.214; $r^2 = 0.70$; N = 19; *L. elliptica* (this study and Ahn and Shim, 1998): log R = 0.888 * log (mg AFDM) -0.436; $r^2 = 0.777$; N = 74. Intercepts differed significantly (p = 0.023, ANCOVA) but slopes did not.

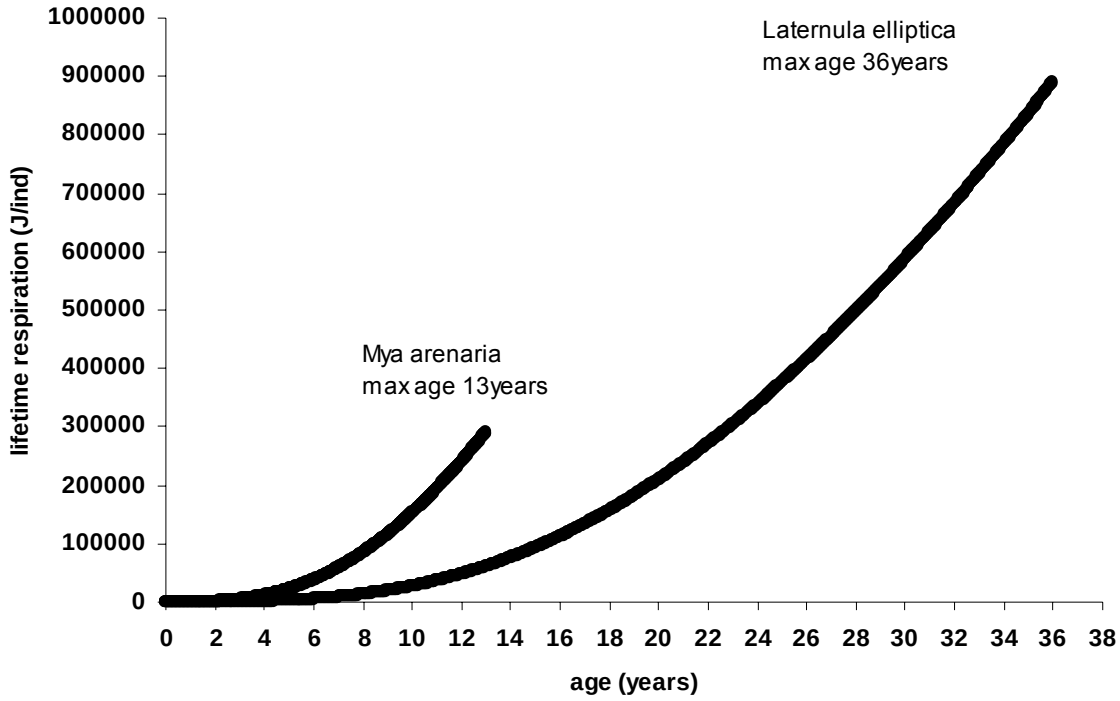

Fig. 3: Lifetime aerobic expenses of *M. arenaria* and *L. elliptica* were computed from the corresponding models shown in Table 1. AFDM was converted to Joule (J) by a conversion factor of 21.465 J/mgAFDM for *M. arenaria* and 20.37 J/mgAFDM for *L. elliptica* from compiled data by T. Brey (unpublished).

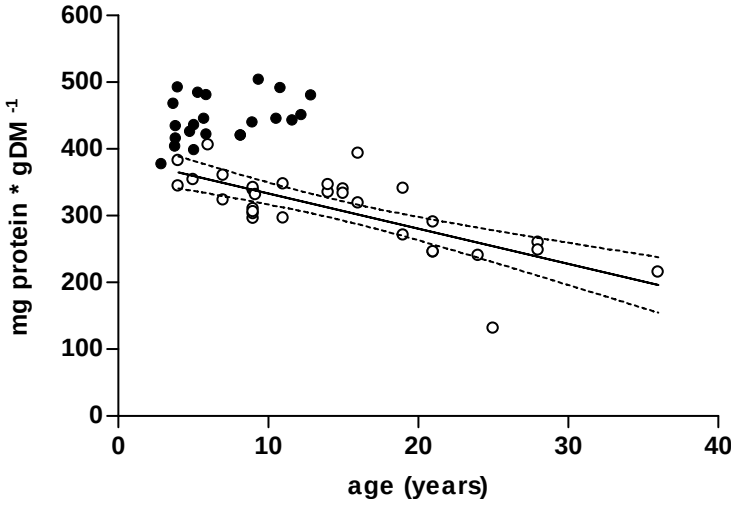

Fig. 4: Protein content (mg protein g^{-1} dry mass) in mantle tissue of *M. arenaria* (filled circles, N = 22, age range = 3-13, mass range = 84-3368 mg AFDM) and *L. elliptica* (open circles, N = 31, age range = 4-36, mass range = 433-3062 mg AFDM) plotted against chronological age (years). Data represent means of duplicate measurements. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *L. elliptica*: Protein content = -5.27 * age + 386.0; $r^2 = 0.55$; N = 31; *M. arenaria*: No significant relationship (slope = 0).

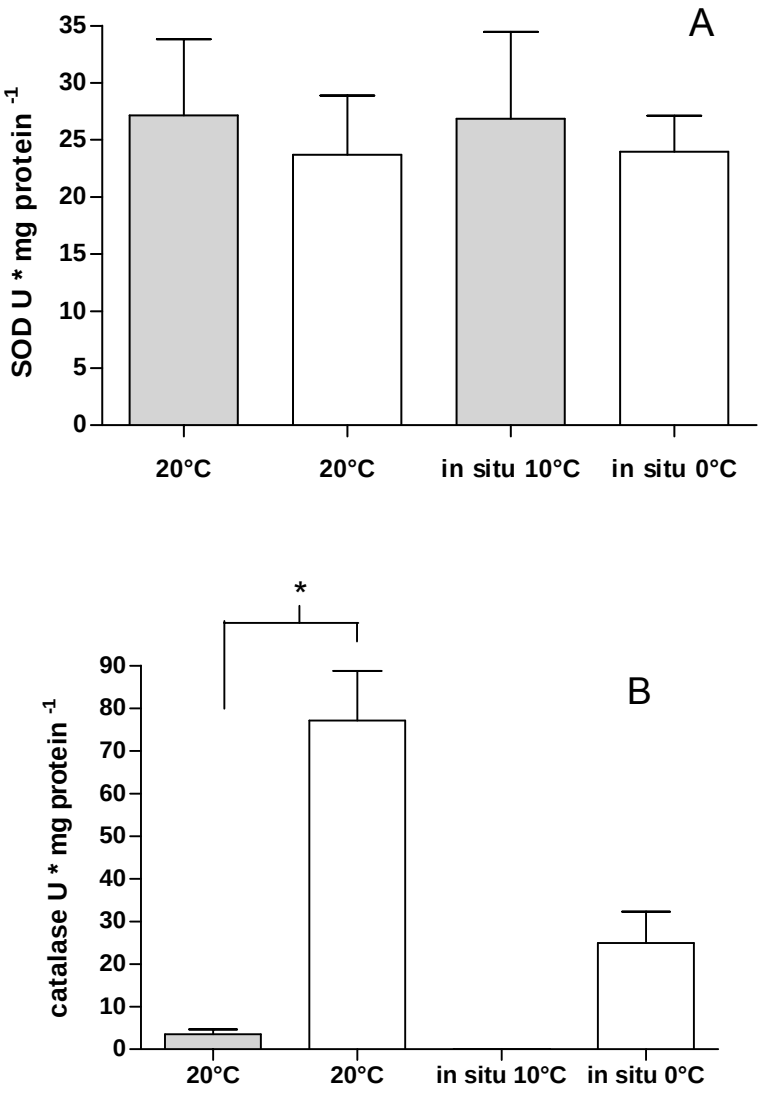

Fig. 5: Activities of superoxide-dismutase (A) and catalase (B) in mantle tissue of *M. arenaria* (M.a., grey bars) and *L. elliptica* (L.e., white bars) at 20°C reference temperature and at *in situ* temperature for both species, 10°C (M.a.) and 0°C (L.e.). Data are means ± SD expressed as U mg^{-1} protein as average over all ages. Age of *M. arenaria* ranged between 2-8 years (mass range = 47-1187 mg AFDM) and of *L. elliptica* 2-28 years (mass range = 95-3278 mg AFDM) (2-36 years for L.e. SOD, mass range = 122-3278 mg AFDM). * = significant different with $p < 0.001$ (students t-test). N catalase, (SOD) = 16 (17) (M.a.) and 29 (38-44) (L.e.).

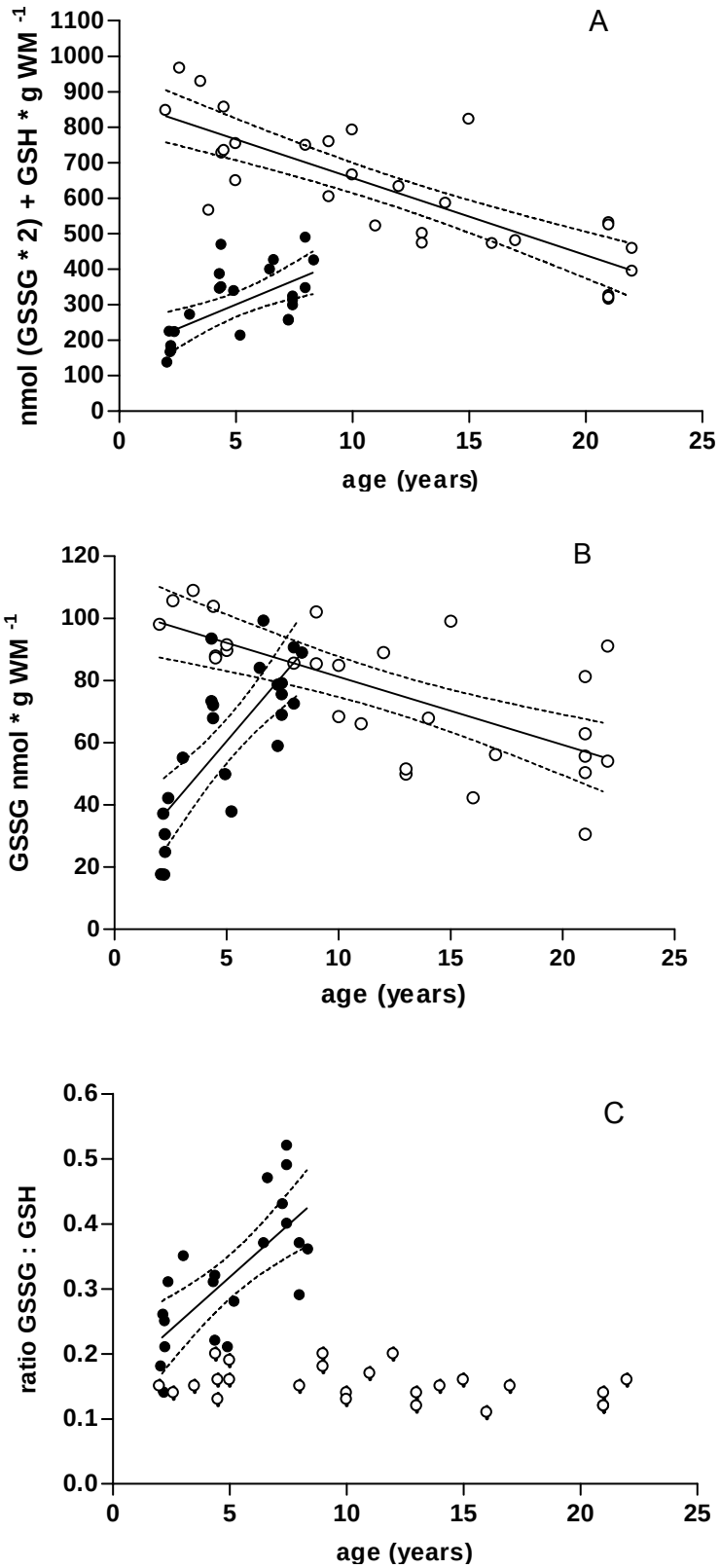

Fig. 6: Concentration of (A) total (tGSH =2*GSSG+GSH), (B) oxidised glutathione (GSSG), and (C) the ratio of oxidised to reduced glutathione (GSSG/GSH) in mantle tissue of *M. arenaria* (filled circles, N = 23, for ratio N = 21; age range = 2-8 years, mass range = 42-1187 mg AFDM) and *L. elliptica* (open circles, tGSH N = 29, GSSG N = 28, for ratio N = 25; age range = 2-22 years; mass range = 100-2563 mg AFDM) vs. chronological age. Data represent means of duplicate

measurements of 1 to 4 pooled animals. Slopes differed significantly between species ($p < 0.001$, ANCOVA).

tGSH: *M. arenaria*: tGSH = 27.67 * age + 171,4; $r^2 = 0.38$; N = 23, p = 0.0017; *L. elliptica*: tGSH = -21.71* age + 874.2; $r^2 = 0.66$; N = 29, p<0.0016. GSSG: *M. arenaria*: GSSG = 8.57 * age + 20.73; $r^2 = 0.599$; N = 23, $p < 0.001$, *L. elliptica*: GSSG = -2.190 * age + 103.2; $r^2 = 0.47$; N = 28, $p < 0.001$. GSSG:GSH: *M. arenaria*: GSSG:GSH = 0.0324 * age + 0.168; $r^2 = 0.52$; N = 21, $p < 0.001$; *L. elliptica*: No significant relationship (slope = 0).

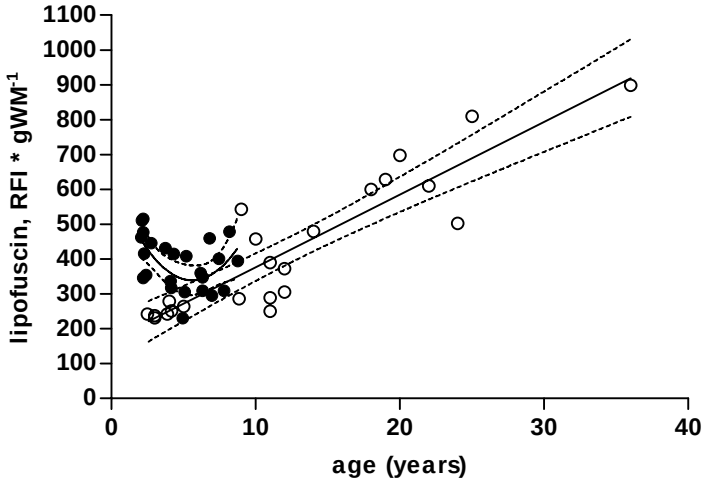

Fig. 7: Lipofuscin content (relative fluorescence intensity (RFI) g^{-1} wett mass) vs. chronological age (years) in mantle tissue of *M. arenaria* (filled circles, N = 24, age range = 2-8 years, mass range = 42-1187 mg AFDM) and *L. elliptica* (open circles, N = 23, age range = 2.5-36 years, mass range = 135-3279 mg AFDM). *L. elliptica*: lipofuscin = 20.88 * age + 167.5; $r^2 = 0.82$; N = 23, $p < 0.05$. The relation between lipofuscin and age for *M. arenaria* was descibed best by the polynome: lipofuscin = 592.9 + (-97.83) * age + 9.383 * age^2 , $r^2 = 0.334$, N = 24, p function = 0.0127, p intercept < 0.001, p slope 1 = 0.0226, p slope 2 = 0.0126.

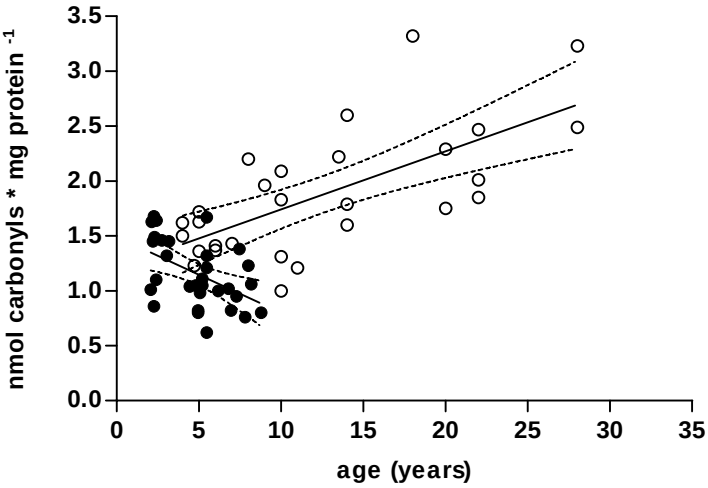

Fig. 8: Protein carbonyl content (nmol mg^{-1} protein) in mantle tissue of *M. arenaria* (filled circles, N = 31, age range = 2-8 years, mass range = 42-1187 mg AFDM) and *L. elliptica* (open circles, N = 28, age range = 4-28, mass range = 227-3506 mg AFDM) vs. chronological age (years). Each data point refers to one individual. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *M. arenaria*: protein carbonyls = -0.0704 * age + 1.472; $r^2 = 0.24$; N = 23, $p = 0.005$. *L. elliptica*: protein carbonyls = 0.052 * age + 1.213; $r^2 = 0.45$; N = 28, $p < 0.001$

Publication II

Mitochondrial ageing of a polar and a temperate mud clam

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Abstract

We investigated mitochondrial ageing in a temperate (*Mya arenaria*) and an Antarctic (*Laternula elliptica*) mud clam, with similar lifestyle (benthic filter feeders) but different maximum life spans (MLSP), 13 years and 36 years respectively. The short-lived temperate *M. arenaria* showed a more pronounced decrease in mitochondrial function (respiration, respiratory control ratio, proton leak, membrane potential) with age than the long-lived Antarctic *L. elliptica.* H_2O_2 generation rates at habitat temperature were far higher in the short-lived *M. arenaria* compared to *L. elliptica*. Reactive oxygen species (ROS) production as proportion of the mitochondrial oxygen consumption rate (%H_2O_2/O_2) increased significantly with age in *M. arenaria*, whereas in *L. elliptica* the proportion remained unchanged. Lower rates of mitochondrial H_2O_2 generation were presumably due to mild uncoupling as *L. elliptica* mitochondria showed higher proton leak compared to *M. arenaria* mitochondria. The results are discussed in to the light of the “Free Radical-Rate of Living theory”, (Pearl, 1928; Harman, 1956) and the “Uncoupling to Survive” hypothesis (Brand, 2000).

1. Introduction

With the exception of some simple forms like hydra, planarians and turbellarians (Child, 1915; Balazs and Burg, 1962; Haranghy and Balazs, 1964; Martinez, 1998), all multicellular organisms age. Two theories link the process of ageing and the maximum life span (MLSP) of a species to mitochondrial oxygen free radical (ROS) formation.

(i) The “Free Radical-Rate of Living theory”, (Pearl, 1928; Harman, 1956) predicts a negative correlation between SMR and MLSP due to increased mitochondrial production of oxygen free radicals at higher standard metabolic rate (SMR) (Ku et al., 1993). (ii) The “uncoupling to survive” hypothesis (Brand, 2000) is based on the same assumption of a negative correlation between ROS production and MLSP, but further predicts that mitochondrial uncoupling mechanisms may modulate reactive oxygen species (ROS) production, altering the strict dependency of ROS formation on SMR.

When comparing marine and freshwater ectotherms of similar lifestyle, several groups discovered higher MLSPs in species from permanently cold compared to temperate environments (Brey, 1991; Brey et al., 1995; Ziuganov et al., 2000; Cailliet et al., 2001; La Mesa and Vacchi, 2001). In Antarctica, marine ectotherms experience year round permanent cold temperatures between -1.9 and +2.0°C and most species have lower SMRs than related species from temperate environments, where temperatures fluctuate from 0 to 18°C (Clarke, 1983; Heilmayer et al., 2004). According to the “Free Radical-Rate of Living theory “, these lower SMRs and the correspondingly low ROS generation by aerobic mitochondrial activity might result in a slow-down of physiological ageing and may explain the higher MLSP of polar ectotherms compared to their temperate relatives. However, aside from higher mitochondrial densities in several polar (Johnston et al., 1998) and sub-polar species (Sommer and Pörtner, 2002), mitochondrial adaptations in the cold involve higher cristae densities and altered membrane fatty acid composition, that may modulate free radical leakage from mitochondria and compromise the simple relationship between SMR and MLSP (Archer and Johnston, 1991; Johnston et al., 1994; St.-Pierre et al., 1998; Sommer and Pörtner, 2002).

For an analysis of whether MLSP is set by the level of SMR and by associated differences in mitochondrial functioning in marine ectotherms, we investigated age dependent changes of mitochondrial energy coupling and ROS formation in isolated mitochondria from mantle tissue of the Antarctic mud clam *Laternula elliptica* (Pholadomyoida) and the North Sea mud clam *Mya arenaria* (Myoida). Both clams are representatives of the same ecotype (benthic filter feeders and burrowing clams) and important key species in their respective habitat, but have adapted to different temperature regimes over long evolutionary time scales (Soot-Ryen, 1952; Petersen et al., 1992; Jonkers, 1999). With a

maximum age of approximately 36 years, *L. elliptica* has a 3-fold longer MLSP than *M. arenaria* with ~13 years MLSP. Although the animals belong to different bivalve subclasses their similarity in size, morphology and lifestyle should justify a comparison of physiological ageing parameters between both species.

2. Material and methods

2.1. Sampling and maintenance

2.1.1. Laternula elliptica

Antarctic *Laternula elliptica* were collected by divers in the Potter Cove, King George Island, South Shetland Island (62°14`S, 58°40`W) in November-February 2002/2003 at 5 to 10 m depths, 34 PSU and temperatures between -1 to +2°C. Animals were maintained in aquaria with seawater from the cove at 0°C for several days until they were used for experimentation. Water was exchanged once a week to ensure a good water quality and food supply. All measurements were carried out at the Dallmann-Laboratory, King George Island, Antarctica.

2.1.2. Mya arenaria

North Sea *Mya arenaria* were sampled with a box corer at a shallow subtidal muddy site close to Harlingen (53°09`N, 05°19´E), The Netherlands, in summer 2002 and 2003. At the Alfred-Wegener-Institute, animals were kept in aquaria with sediment at 10°C seawater and 28 PSU salinity. Animals were fed plankton tablets (REWE, Germany) twice a week. All measurements were undertaken at the Alfred-Wegener Institute for Polar- and Marine Research in Bremerhaven, Germany.

2.2. Age determination

Age determination for *M. arenaria* was done using a Von Bertalanffy (1934) growth model (VBGF) based on length-at-age data of the same subtidal population:

$$St = 117.17 * (1-e^{-0.12 * (t + 0.054)})$$

Due to the high variability in the relation between shell length and age, *L. elliptica* were individually aged by individual ring counts in polished cuts through the shell umbo (Brey and Mackensen, 1997). For a more detailed description see Philipp et al. (2005 in press).

2.3. Mitochondrial measurements

2.3.1. Isolation of mitochondria

Mitochondria were isolated from the mantle tissue of freshly sacrificed bivalves. Depending on size, tissues of 1-9 individuals of *L. elliptica* and up to 22 *M. arenaria* specimens were pooled for one experiment. About 4g of mantle tissue was finely chopped in 10 ml ice cold homogenisation buffer (400 mM sucrose, 70 mM Hepes, 100 mM KCl, 3 mM EDTA, 6 mM EGTA, 1% bovine serum albumine, 1µl/ml aprotinine, pH 7.3) modified after Moyes et al. (1985) and Heise et al. (2003).

Briefly, the tissue was homogenised in a pre-cooled glass/teflon-homogeniser, the homogenate centrifuged at 1300xg for 15 min at 2°C and the supernatant collected. The pellet was resuspended, homogenised, and again, mitochondria extracted at the same speed. The two supernatants were combined and centrifuged at 10500g for 15 min to sediment the mitochondria. The resulting mitochondrial pellet was resuspended in 1.5-2 ml assay medium (560 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 70 mM Hepes, 5 mM glutamate, 1µg/ml aprotinine and 1% bovine serum albumine at pH 7.3).

2.3.2. Respiration and membrane potential of isolated mitochondria

Measurements were carried out using a water-jacketed respiration chamber under gentle stirring. Mitochondrial respiration was recorded by oxygen microoptodes (TX PreSens GmbH, Neuweiler, Germany) and membrane potential determined using TPMP-sensitive electrodes according to Brand (1995). Measurements were carried out at 0°C for *L. elliptica* and 10°C for *M. arenaria* mitochondria. Oxygen concentrations were calculated using the oxygen solubility ($ßO_2$) according to Johnston et al. (1994) and the atmospheric pressure of the day. For mitochondrial respiration and membrane potential measurements 5 mM succinate was added as substrate and 5µM rotenone to prevent respiration of endogenous NAD-linked substrates (Brand, 1995). State 3 respiration was induced by addition of 0.075-0.15 mM (*M. arenaria*) and 0.06-0.09 mM (*L. elliptica*) ADP. Non-phosphorylating respiration, comprising oxygen consumption by proton leak and ROS formation (state 4+), was recorded after adding 2µg/ml, of the F_0F_1-ATPase inhibitor oligomycin. The respiratory control ratio (RCR), which describes how effectively the respiratory chain is coupled to the ATPase, was calculated according to Estabrook (1967), using state 4+ respiration. The percentage of proton leak of state 3 oxygen consumption was calculated as (state 4+/state 3) *100. Membrane potential measurements are described in detail in Keller et al. (2004).

2.3.3 Production of hydrogen peroxide (H_2O_2) by isolated mitochondria.

Mitochondrial hydrogen peroxide production was measured fluorimetrically ($\lambda_{excitation}$= 312 nm and $\lambda_{emission}$ = 420 nm) recording the reaction of H_2O_2 with homovanilic acid (HVA) in the presence of horse radish peroxidase (HRP) modified after Miwa et al. (2003). Measurements of the H_2O_2 generation rate of *L. elliptica* mitochondria were performed with a Shimadzu (RF-1501) fluorometer at the Dallmann Laboratory, whereas *M. arenaria* mitochondria were measured with a Perkin Elmer (LS 50B) fluorometer in Bremerhaven. Incubation of 275 µl *L. elliptica* mitochondrial suspension in 775µl assay medium, containing 10 µM rotenone, 0.3 mM HVA, 6.5 U/ml HRP was performed in a water-jacketed respiration chamber under gentle stirring outside the fluorometer at 0°C. Initial fluorescence recordings on a 2 channel chart recorder (Kipp & Zonen, Netherlands) were run after adding 200µl of the incubated mitochondrial mixture to 800µl phosphate buffer (10 mM KH_2PO_4, 100 mM Na_2HPO_4, pH 7.1). After 45 min of state 2 respiration with succinate, fluorescence values were recorded again (200µl incubated mitochondrial suspension plus 800µl phosphate buffer) and the difference to baseline fluorescence was calculated. H_2O_2 generation in state 3 was measured after adding ADP to the incubated mitochondria. In each experiment, fluorescence was calibrated with an H_2O_2 standard (0.2 nM; Merck, Germany). Each value was determined with 2-3 replicate measurements and the whole experiment was repeated at least twice with every mitochondrial isolate. Controls run without mitochondria, showed no fluorescence produced under any assay condition.

Using the LS 50B Perkin Elmer fluorometer with a cooled sample compartment and magnetic stirring, the H_2O_2 generation in states 2 to 4+ were recorded for one and the same mitochondrial aliquot directly in the fluorometer. 150µl *M. arenaria* mitochondrial solution was incubated with 850µl assay medium, with the following chemicals added in the order: 5µM rotenone, 5µM of the myokinase inhibitor Ap5A (P^1, P^5-adenosine-5´-pentaphosphate), 0.1 mM HVA, 2.5 U/ml HRP at 10°C. When a steady fluorescence signal was reached, succinate (state 2), ADP (state 3) and oligomycin (state 4+) were added following the same protocol as in the respiration measurements. Again each measurement was calibrated with an H_2O_2 standard.

A comparison with both fluorometers was run on *M. arenaria* mitochondria to assure that methodological effects did not compromise the results. Both, H_2O_2 generation rates and oxygen consumption rates were measured in parallel and related to mitochondrial protein (Keller et al., 2004).

2.4. Enzyme assays

For enzyme assays animals were rapidly dissected and mantle tissue freeze clamped and stored in liquid nitrogen until processing. Assay temperature for photometric measurements was set to *in situ* temperature for both animals, 0°C for *L. elliptica* and 10°C for *M. arenaria*, and 20°C for both species as a reference temperature. Assays were run at least in duplicate for each sample and results expressed as international units (µmol of substrate converted to product min $^{-1}$) mg $^{-1}$ protein. Protein content was determined with the Biuret method modified after Kresze (1988).

2.4.1. Cytochrome c oxidase (COX) and citrate synthase (CS) measurements

For COX (EC 1.9.3.1) and CS (EC 4.1.3.7) measurements, frozen mantle tissue was ground in liquid nitrogen and homogenised with a glass homogeniser (Nalgene, USA) in Tris-HCl buffer (20 mM Tris-HCL, 1 mM EDTA, 0.1% (v/v) Tween® 20, pH 7.4) 1:3 (w/v) for COX, and 1:4 (w/v) for CS.

For COX measurements, homogenates were centrifuged for 10 min at 1000xg and 2°C. COX activity was determined after Moyes (1997) by measuring the oxidation rate of cytochrome c at 550 nm in 20 mM Tris-HCL buffer with 0.5% Tween 20, pH 8.0 Activity was calculated using the mmolar extinction coefficient ε_{550}mM 19.1 mM $^{-1}$ cm^{-1} after Hardewig et al (1999).

Homogenates for CS activity were sonicated for 15 min in Branson Sonifier 450 (output control 8, Duty cycle 50%) cooled to 0°C and centrifuged at 7400xg for 5 min at 2°C. CS activity was measured after Sidell et al. (1987) recording the absorbance increase of 5 mM DTNB (5,5´dithiobis(2-nitrobenzoic acid)) in 100 mM Tris-HCL (pH 8.0), 20 mM Acetyl-CoA and 20 mM Oxaloacetat at 412 nm. Activity was calculated using the mmolar extinction coefficient ε_{412} of 13.61 mM $^{-1}$ cm^{-1}.

The temperature coefficient Q_{10} was calculated as:

$$Q_{10} = e^{10\,(dlnEA/dT)}$$

With dlnEA being the difference in ln enzyme activity at the higher and lower temperature and dT being the difference between assay temperatures.

2.5. Calculations and statistics

Analyses of variance (ANOVA) and covariance (ANCOVA) were used to analyse the relationship between parameters vs. age and to identify differences between species after testing the data for normality. Data of mitochondrial respiration were $\log_{10}$ transformed for linearization. Lines shown in graphs are regression lines and the upper and lower 95% confidence bands.

3. Results

3.1. Age dependent changes in the function of isolated mitochondria

Oxygen consumption of mitochondria isolated from mantle tissue of the temperate *M. arenaria* and the polar mud clam *L. elliptica* at *in situ* temperatures are presented in Fig. 1.

A significant decline of respiratory capacity with chronological age was recorded in both species. The slope of the decrement was, however, two times steeper in *M. arenaria* than in *L. elliptica* mitochondria. This resulted in lower respiration rates of mitochondria isolated from aged *M. arenaria* compared to aged *L. elliptica* mitochondria. Coupling of respiration to mitochondrial phosphorylation was best in isolated mitochondria of young *M. arenaria* and decreased significantly with age (Fig. 2). Mitochondria isolated from *L. elliptica* had generally lower RCRs than *M. arenaria* mitochondria. RCRs decreased significantly with age in both species, however, again the change was more pronounced in *M. arenaria*. The non-phosphorylating leak of protons into the mitochondrial matrix increased significantly with age in both species (Fig. 3). In the temperate species the age related increase in proton leakage was significantly more rapid compared to the polar species, when plotted against chronological age. Generally, isolated mitochondria from the polar mud clam had a higher proton leak than *M. arenaria* mitochondria (Fig. 3).

Mitochondrial membrane potential ($\Delta\Psi$) of state 4+ respiration with nigericin declined significantly with age in *M. arenaria,* whereas in *L. elliptica* $\Delta\Psi$ remained stable around 134 mV (SD 8.202 mV) with no age-dependent variations (Fig. 4).

The generation of hydrogen peroxide (H_2O_2) in state 2 with succinate was measured in isolated mitochondria of both species and is presented in Fig. 5 on a mg protein basis. Isolated mitochondria from young *M. arenaria* produced more than 3 times the amount of H_2O_2 per mg protein (0.1 nmol H_2O_2 mg^{-1} protein) than *L. elliptica* mantle mitochondria (0.03 nmol H_2O_2 mg^{-1} protein). H_2O_2 generation per mg mitochondrial protein with age was constant in *M. arenaria*, whereas in *L. elliptica* a significant decrease with age was observed.

The proportion of ROS production of the mitochondrial oxygen consumption rate $\%H_2O_2/O_2$ [((nmol H_2O_2 mg $protein^{-1}$ min^{-1}/nmol O_2 mg $protein^{-1}$ min^{-1})/100) * 2, for a detailed description see Keller et al., (2004)] could be calculated for states 3 and 2. State 2 equals state 4 in the sense that ADP is absent and the F_0F_1 ATPase is not inhibited by oligomycin. Under non-phosphorylating conditions (state 2), a far higher fraction of up to 6 times more oxygen was converted to ROS in mitochondria isolated from *M. arenaria* than from *L. elliptica* (Fig. 6A). The $\%H_2O_2/O_2$ increased significantly with age in mitochondrial

isolates from the temperate species, whereas in the polar species the percent ROS production was generally low, and moreover decreased mildly with age. $\%H_2O_2/O_2$ during maximal energetic coupling in state 3 was extremely low in both species (Fig. 6B).

3.2. Activities of mitochondrial enzymes

Table 1 lists the activities of the mitochondrial enzymes citrate synthase (CS) and cytochrome c oxidase (COX) in the mantle tissue of *M. arenaria* and *L. elliptica*, at reference and at *in situ* temperature. At reference temperature, *L. elliptica* displayed higher CS and COX activities per mg protein than *M. arenaria*. Even at *in situ* temperature COX activities were higher in *L. elliptica* compared to *M. arenaria*, whereas CS activities were higher in *M. arenaria* (Table 1). No significant change in enzyme activity with age was found in either species. COX Q_{10} values were the same in both species 2.92 +/- 0.57 (*L. elliptica*) and 2.53 +/- 0.87 (*M. arenaria*), but for CS *L. elliptica* displayed a higher Q_{10} (2.8 +/- 0.42) than *M. arenaria* (1.83 +/- 0.15).

4. Discussion

The present study clearly demonstrates age related changes of mitochondrial functions in both investigated bivalves. This is in line with other studies which found important changes in isolated mitochondria from aging humans (Trounce et al., 1989; Cooper et al., 1992; Boffoli et al., 1994), other vertebrates like rats (Nohl and Hegner, 1978; Ventura et al., 2002) and invertebrates (Sohal et al., 1995), see Shigenaga et al. (1994) for review. Moreover, Hagen et al. (1997) documented that mitochondrial functions not only decline when investigated in isolated mitochondria, but also in isolated cells of aging rats.

In both investigated mud clams respiratory capacities and RCRs of isolated mitochondria decreased, whereas proton leak increased with age. Both, magnitude and timescale of these changes, however, differ strikingly between both species. In the shorter-lived temperate mud clam age-related changes proceed more rapidly than in the longer-lived polar species.

The results can also be plotted against relative age, i.e. the percentage of an age t at the maximal age of the species. Relative age was calculated by using the maximum age found within the species population and assuming this to be the maximum age the species can attain. This assumption is supported by the literature for the different species (Ralph and Maxwell, 1977; Winther and Gray, 1985; Urban and Mercuri, 1998; Strasser, 1999) and additionally for *L. elliptica* by over 650 individual age determinations of animals sampled around King George Island (this study, M. Voigt (2004), Urban et al., (1998) and T. Brey unpublished data). When plotted against relative age (Fig. 7), species specific

slopes of respiratory capacity and RCR were still significantly higher in *M. arenaria* than in *L. elliptica*. This indicates that mitochondrial ageing is not only faster but also more pronounced in the temperate than the polar species.

We hypothesized that an explanation for accelerated ageing in *M. arenaria* could be the higher rates of mitochondrial ROS formation. H_2O_2 generation per milligram mitochondrial protein was far lower in the polar mud clam, although protein specific respiration rates at *in situ* temperature were similar or even higher in *L. elliptica* mitochondria. Along with decreasing rates of mitochondrial respiration, H_2O_2 generation declined significantly in aged *L. elliptica* individuals. The clear-cut relationship between mitochondrial ROS generation and respiratory rate is still subject to controversy (Barja, 1999). In our study *M. arenaria* mitochondrial respiration rates declined dramatically with age, whereas H_2O_2 generation per milligram mitochondrial protein remained constant, because a higher percentage of consumed oxygen was converted to H_2O_2 ($\%H_2O_2/O_2$) in mitochondria of aged compared to younger *M. arenaria*. In the longer-lived *L. elliptica* no significant change in $\%H_2O_2/O_2$ with age was found and the overall $\%H_2O_2/O_2$ ratio was far lower than in the shorter-living *M. arenaria*. Our findings agree with a study comparing $\%H_2O_2/O_2$ of isolated mitochondria from pigeon and rats with different MLSPs, in which isolated mitochondria of long-lived pigeons showed significantly lower $\%H_2O_2/O_2$ than mitochondria from the shorter lived rats (Barja et al., 1994).

It remains open, which mitochondrial mechanism causes the higher H_2O_2 generation rates in *M. arenaria* and what causes the increase in $\%H_2O_2/O_2$ with age. A basic difference is the comparably higher proton leak in isolated mitochondria of the polar *L. elliptica* compared to the temperate *M. arenaria* which, according to Brand (2000), could account for the lower ROS generation in *L. elliptica*. According to Porter et al. (1996), proton leak increases as a function of inner mitochondrial membrane surface area (cristae density) and can be modulated by the degree of unsaturated fatty acids in the membrane (Porter et al., 1996). Increased cristae density has already been detected in active rainbow trout following cold acclimation (St.-Pierre et al., 1998). An elevated content of unsaturated membrane fatty acids is common in marine fish from low latitudes (Cossins et al., 1978; White and Somero, 1982) and held responsible for the higher proton leak in some subpolar species (Guderley, 2004).

Whether either mechanism accounts for the higher proton leak rate in the polar mud clam remains speculation. However, Ahn et al. (2000) reported similar levels of total unsaturated fatty acids in the soft tissue of *L. elliptica* and several marine bivalves from warmer waters, raising some doubt as to the involvement of membrane unsaturation in elevated proton leak rates in the polar clam. An indication for potentially higher cristae

density in *L. elliptica* than in *M. arenaria* mitochondria may be seen in the 3-fold higher activity (U per mg protein at *in situ* temperature) of cytochrome c oxidase (COX), the enzyme being located in the mitochondrial inner membrane. In contrast, the activity of matrix located citrate synthase (CS) was slightly higher in *M. arenaria* (Table 1). At reference temperature CS values were 2-fold higher in *L. elliptica* than *M. arenaria*, whereas the difference in COX activities was even more pronounced and 11-fold higher in *L. elliptica*. COX-Q_{10} values were similar in both animals and the higher activities in *L. elliptica* are therefore attributed to higher COX quantities possibly paralleled by higher cristae density, rather than a qualitative difference in enzyme activities between the two species. In line with this, St. Pierre et al. (1998) found significantly higher COX activities (U mg^{-1} mitochondrial protein) in winter compared to summer acclimatised rainbow trout while CS activities (U mg^{-1} mitochondrial protein) were only mildly increased in winter animals. This went along with higher mitochondrial cristae density in winter compared to summer animals. We conjecture that ROS formation in *L. elliptica* is minimized by mild uncoupling (Brand, 2000) due to proton leakage, presumably because of higher cristae density, and that this may be a strategy employed by the polar clams, to prolong mitochondrial and animal lifetime in the cold.

The higher proton leakage (Porter et al., 1996) could explain the higher mitochondrial respiration rates at *in situ* temperature in *L. elliptica* compared to *M. arenaria* in individuals older than 35% of maximum age. In contrast, comparing whole animal respiration, the polar *L. elliptica* displayed more than 2-times lower standard metabolic rates (SMR) than the temperate clam at in-*situ* temperature and at all ages (Philipp et al., 2005 in press). This discrepancy between lower whole animal SMR and higher mitochondrial respiratory capacity in the polar clam could be explainable when assuming lower numbers of highly active mitochondria per g wet mass in *L. elliptica.* These animals may employ two defence strategies to suppress the production of hazardous ROS: a low SMR at *in situ* temperatures (“Free Radical-Rate of Living” theory) and mild uncoupling by an elevated mitochondrial proton leak (“Uncoupling to survive” theory). However, ultrastructural analyses are necessary to confirm our assumptions concerning lower mitochondrial volume and higher cristae density in the polar species.

A correction of the oxygen consumption of *M. arenaria* mitochondria ascribed to proton leak for the amount of oxygen that goes into H_2O_2 formation (Heise et al., 2003) resulted in a lower calculated proton leak (Fig. 8). Net proton leak was calculated after correcting state 3 and 4+ respiration for the percentage of H_2O_2 formed in each state. A similar correction cannot be done for our *L. elliptica* data, as no H_2O_2 measurements of state 4+ mitochondria were performed. However, because of the low overall H_2O_2 generation in

state 2 and stable or even decreasing H_2O_2 generation rates with age, a correction of the proton leak in *L. elliptica* mitochondria can result in only minor changes of the calculated data. The difference in proton leak between *L. elliptica* and *M. arenaria* mitochondria would then be even more apparent.

The significant increase of proton leak with age found prior H_2O_2-correction in *M. arenaria* is abolished when corrected for H_2O_2 production (Fig. 8), so that we conclude that the significant decrease in membrane potential with age in the temperate clam must be attributed to a slow down of mitochondrial respiration and not to a higher proton leak in older animals.

In the present study H_2O_2 generation at complex III was measured in the presence of succinate and rotenone (St-Pierre et al., 2002; Keller et al., 2004). Addition of SOD (50U/ml) doubled H_2O_2 generation in *M. arenaria*. Due to insufficient sample size the SOD effect could not be measured for *L. elliptica*. However, this indicates that at least in *M. arenaria* mitochondria, ROS are generated not only on the matrix, but also on the cytoplasmic side of the mitochondrial inner membrane (St-Pierre et al., 2002). According to Barja (1994), species with high mitochondrial ROS production are more likely to suffer damage of the inner mitochondrial membrane and of the mitochondrial DNA (mt-DNA), located adjacent to the membrane. Assuming that our *in-vitro* measurements reflect *in-vivo* conditions, continued oxygen radical formation on both sides of the membrane in *M. arenaria* mitochondria and consistently higher than in *L. elliptica,* might give rise to elevated oxidative stress and exacerbate membrane and mt-DNA damage in *M. arenaria*. Like in a “vicious cycle” (Lenaz, 1998), this ensues higher rates of incomplete oxygen reduction with age and may eventually accelerate and exacerbate the aging process in the North Sea mud clam.

We have previously reported changes of antioxidant capacities and tissue redox state (ratio of oxidised to reduced glutathione) in mantle tissue of both species with age (Philipp et al., 2005 in press). Tissue redox state was more oxidised in the temperate clam and increased with age, whereas in the polar species a less oxidized tissue redox state was maintained stable throughout all ages. Moreover, *L. elliptica* displayed higher H_2O_2 scavenging capacities (catalase, glutathione) than *M. arenaria*. Taken together, higher H_2O_2 generation rates, the increase in $\%H_2O_2/O_2$ with age in *M. arenaria* mitochondria as well as lower H_2O_2 scavenging ability may explain the higher GSSG: GSH ratio and lower MLSP in the temperate compared to the polar species.

According to Vladimir Skulachev's (2001) “Samurai law of biology” one would expect destruction of the progressively damaged and ROS generating mitochondria in *M. arenaria* in the sense that “it is better to die than to be wrong”. According to the Samurai

law, mitochondria and cells, but also organs or individuals commit suicide (apoptosis) before they turn into “unhopeful monsters” causing detrimental damage in the organism or the population (Skulachev, 2001). In our study, already young *M. arenaria* show higher lipofuscin accumulation than *L. elliptica* and older *M. arenaria* (Philipp et al., 2005 in press), which may be indicative of a higher mitoptotic potential (mitochondrial apoptosis, Skulachev, 2001) in *M. arenaria*, as damaged mitochondria contribute to lipofuscin formation (Miquel, 1998; Brunk and Terman, 2002b). Further degradation of ROS producing mitochondria may be impaired due to the already lipofuscin loaded lysosomes (Terman, 2001; Brunk and Terman, 2002b; Brunk and Terman, 2002a), leaving wasted and ROS generating mitochondria to accumulate in the tissue.

Conclusions

The results from this and a previous study (Philipp et al., 2005 in press) explain a 3-fold higher MLSP found in a polar compared to a temperate mud clam along the lines of two different ageing theories: the “Free Radical-Rate of Living theory “(Pearl, 1928; Harman, 1956) and the “Uncoupling to Survive” hypothesis (Brand, 2000). In line with the first theory, the longer lived polar *L. elliptica* shows lower SMRs than the shorter lived temperate *M. arenaria* (Philipp et al., 2005 in press). On the other hand, isolated mitochondria from *L. elliptica* display similar capacities but far lower H_2O_2 generation rates than those of *M. arenaria*, possibly due to the higher proton leak in the polar mitochondria. The higher H_2O_2 generation in *M. arenaria* mitochondria may account for the more pronounced decrease in mitochondrial function (respiration rate, RCR) with age compared to the polar species. Lower SMR in combination with low mitochondrial H_2O_2 generation and higher antioxidant defence capacities throughout lifetime could explain the longer MLSP in the polar compared to the temperate species.

Acknowledgements

Rob Dekker and the Crew from the RV Navicula from the NIOZ kindly took E. Philipp on several sampling trips to collect *M. arenaria*. Many thanks to the Argentinean Divers, the Station Management of Jubany, to Thomas Brey, Timo Hirse and Oscar Gonzales for logistic as well as scientific support at the Argentinean Antarctic Base Jubany and in Bremerhaven. The study was supported by a student grant of the University of Bremen. We would like to thank three anonymous referees for insightful revision of this paper.

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Tables:

Table 1: Citrate synthase (CS) and cytochrome c oxidase (COX) activity in the mantle tissue of *M. arenaria* and *L. elliptica* over all ages.

species	**citrate synthase** mean (S.D.) $x10^{-2}$	**cytochrome c oxidase** mean +/- S.D. $x10^{-4}$
M.arenaria 20°C	1.8 (0.6)	6.5 (2.4)
L.elliptica 20°C	4.8 (1.3) *	71.5 (19.0) *
M.arenaria in situ 10°C	1.0 (0.4) *	2.6 (1.8)
L.elliptica in situ 0°C	0.6 (0.3)	8.5 (3.7) *

Enzyme activities were assayed at *in situ* temperature and 20°C reference temperature. Data are expressed as international Units per mg protein. Shown are means and S.D. over all ages. Chronological age of *M. arenaria* ranged from 2 to 8 years and from 2 to 30 years for *L. elliptica*. N (N *in situ*) = 17 (17) (M. a) - 35 (34) (L. e) for CS measurements and 16 (16) (M. a) – 37 (37) (L. e) for COX measurements. * = significantly different between species (students t-test, $p < 0.05$).

Figures:

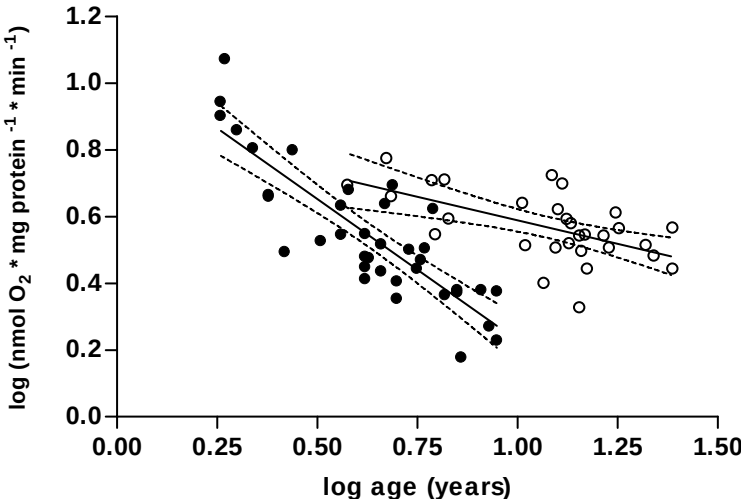

Fig. 1: State 3 respiration of mitochondria isolated from *M. arenaria* (filled circle, N = 37, age range = 2-9 years) and *L. elliptica* (open circles, N = 30, age range = 4-24 years) mantle tissue vs. chronological age. Measurements were carried out at mean *in situ* temperatures (10°C *M. arenaria* and 0°C *L. elliptica*). Each circle represents between 1 and 3 replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *M. arenaria*: $logMO_2 = -0.8470 * log\ age + 1.076$, $r^2 = 0.730$; *L. elliptica*: $logMO_2 = -0.28 * log\ age + 0.87$, $r^2 = 0.344$. For all slopes $p < 0.001$.

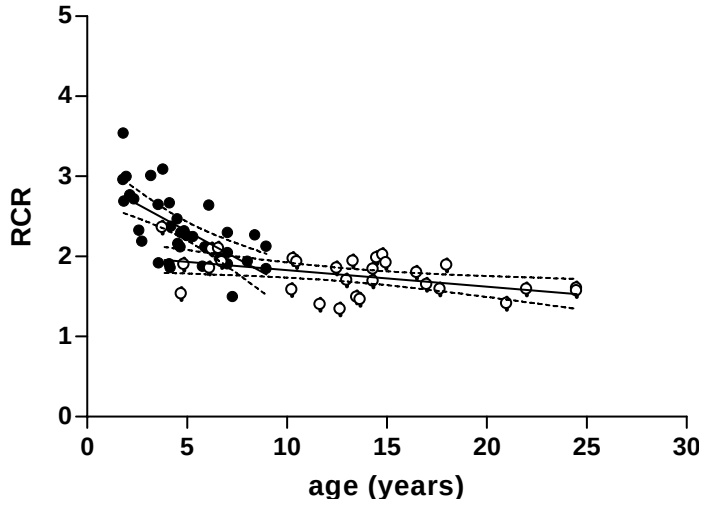

Fig. 2: Respiratory coupling ratio (RCR) of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles, N = 35, age range = 2-9 years) and of *L. elliptica* (open circles, N = 30, age range = 4-24) vs. chronological age (years). Each circle represents between 1 and 3 replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *M. arenaria*: RCR = -0.136 * age (years) + 2.997, $r^2 = 0.428$; *L. elliptica*: RCR = -0.021 * age + 2.04, $r^2 = 0.222$. P values are $p < 0.001$ for *M. arenaria* and $p = 0.009$ for *L. elliptica*.

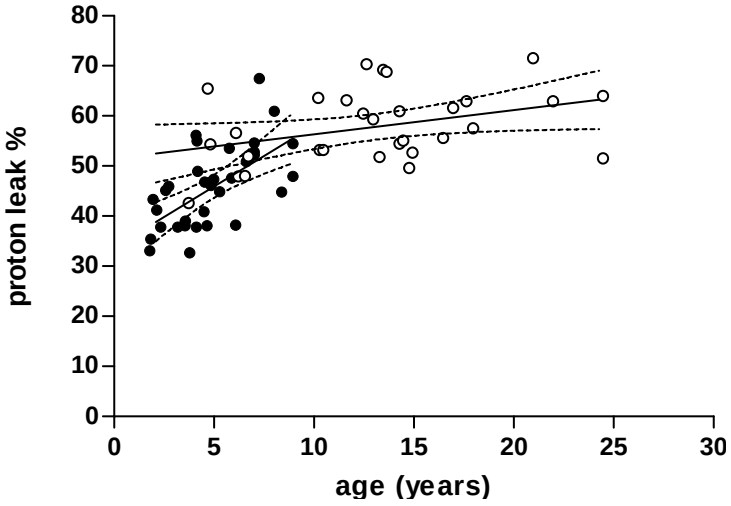

Fig. 3: Percentage of state 4+ in state 3 respiration of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles, N = 33, range = 2-9 years) and *L. elliptica* (open circles, N = 30, age range = 4-24 years) vs. chronological age (years). Each circle represents between 1 and 3 replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.005$, ANCOVA). *M. arenaria*: Leak = 2.4 * age + 33.7, $r^2 = 0.394$, *L. elliptica*: Leak = 0.48 * age + 51.5, $r^2 = 0.137$. $p < 0.001$ for *M. arenaria* and $p = 0.044$ for *L. elliptica* (ANOVA).

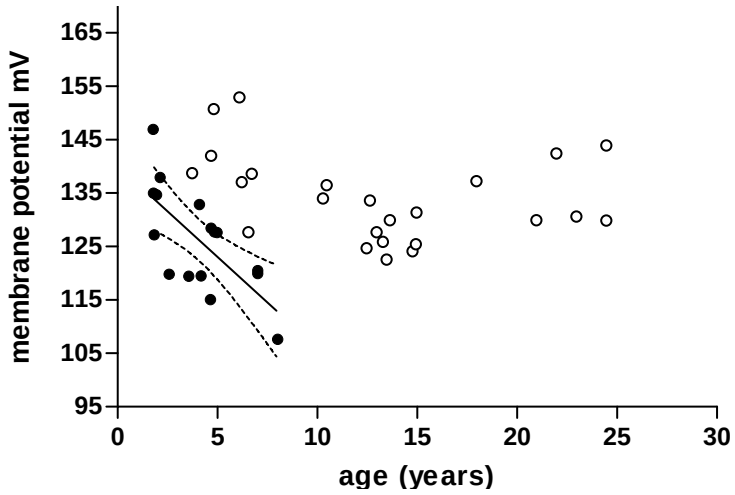

Fig.4: Membrane potential of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles, N = 16, range = 2-8 years) and *L. elliptica* (open circles, N = 24, age range = 4-25 years) vs. chronological age (years) measured at *in situ* temperature. Each circle represents between 1 and 3 replicate measurements per mitochondrial isolation. Slopes differed significantly between species (p = 0.0051, ANCOVA). *M. arenaria*: membrane potential = -5.404 * age – 131.2, r^2 = 0.495, p = 0.0023; *L. elliptica*: no significant relationship.

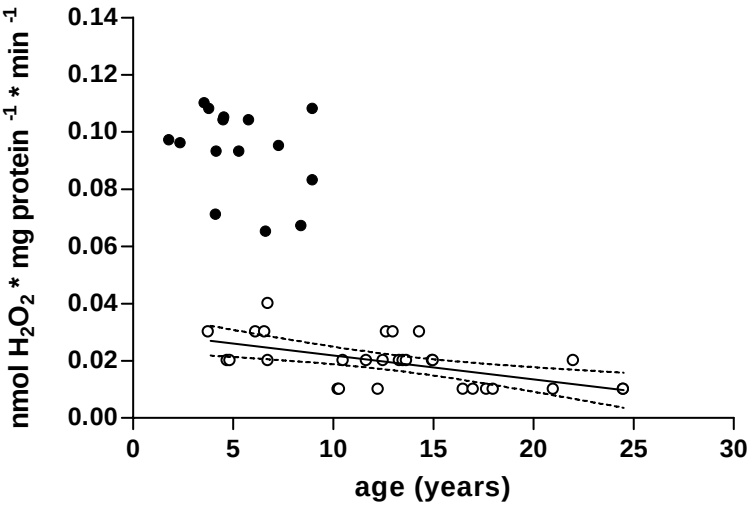

Fig. 5: Generation of hydrogen peroxide (H_2O_2) of isolated mitochondria from mantle tissue of *M. arenaria* (filled circles, N = 15, age range = 3-9 years) and *L. elliptica* (open circle, N = 29, age range = 4-24 years) over chronological age in state 2. Measurements were carried out at mean *in situ* temperatures (10°C *M. arenaria*, 0°C *L. elliptica*). Each circle represents between 1 and 3 replicate measurements per mitochondrial isolation. Slopes of both species were not, but intercepts were significantly difference with p < 0.001 (ANCOVA). *L. elliptica*: H_2O_2 = -0.0008 * age + 0.030, r^2 = 0.320, p = 0.0014; *M. arenaria* = no significant relationship.

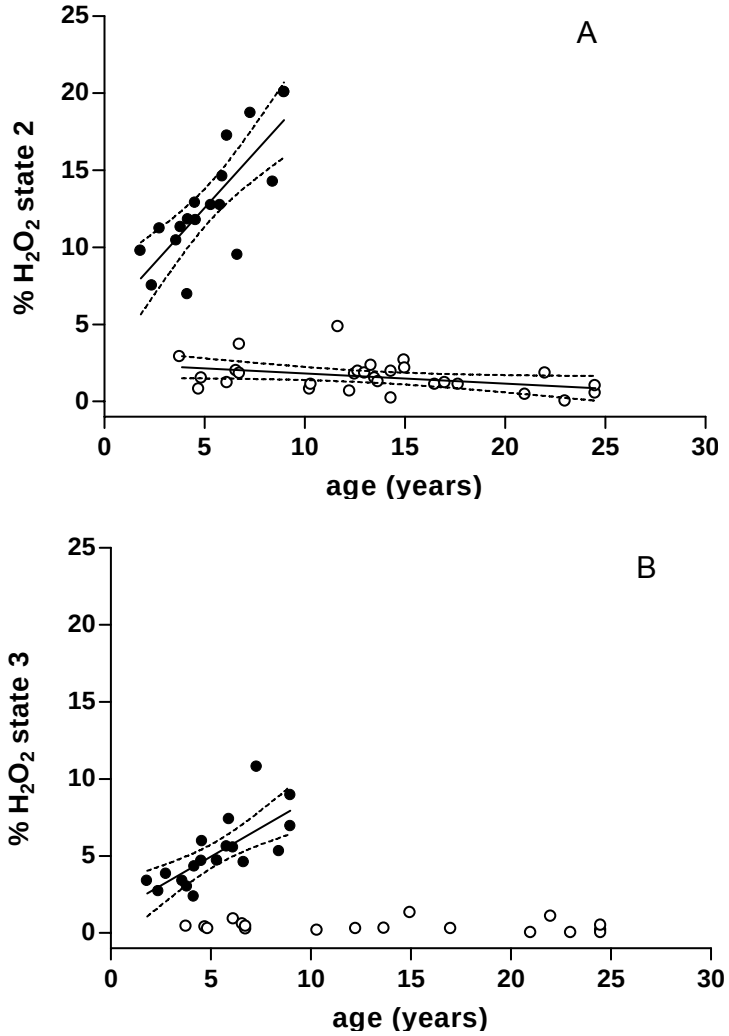

Fig. 6: Percentage of H_2O_2 production in oxygen consumption in state 2 (A) and state 3 (B) of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles) and *L. elliptica* (open circles) depending on chronological age. Measurements were carried out at mean *in situ* temperatures (10°C *M. arenaria* and 0°C *L. elliptica*). Each circle represents between 1 and 3 replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA).A : *M. arenaria*: $\%H_2O_2 = 1.44 * age + 5.33$, $r^2 = 0.637$,N = 18, age range = 3-9 years, $p < 0.001$; *L. elliptica*: $\%H_2O_2 = -0.067 * age + 2.5$, $r^2 = 0.145$, N = 29, age range = 4-24 years, $p = 0.041$; B: M. arenaria: $\%H_2O_2 = 0.755 * age + 1.171$, $r^2 = 0.547$, N = 18, age range = 3-9 years, $p < 0.001$; *L. elliptica*: no significant relationship (N = 18, age range = 4-24 years).

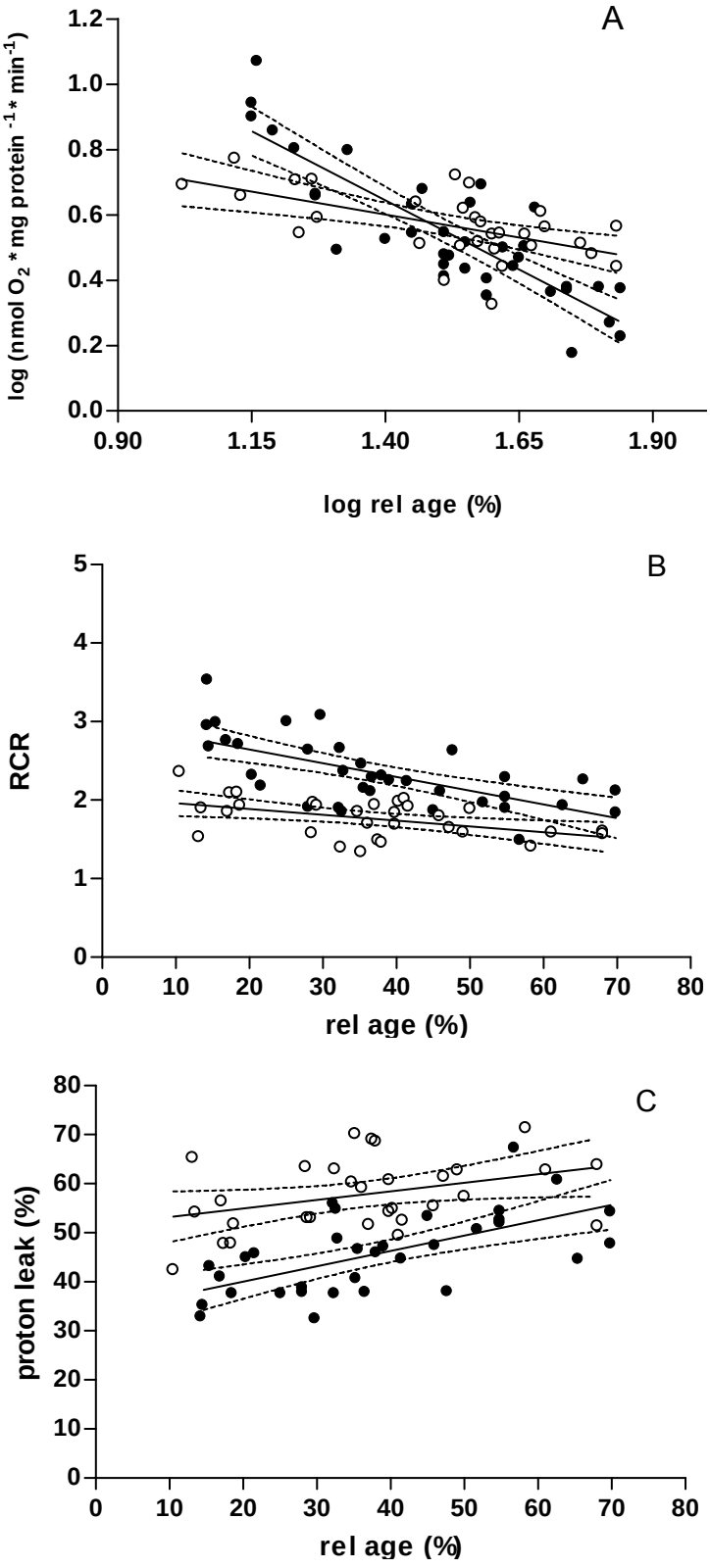

Fig. 7: Functions of isolated mitochondria A) State 3 respiration B) RCR and C) proton leak vs. relative (rel.) age. Legend details and p values for the individual slopes see Fig. 1, 2, 3 respectively. Slopes differed significantly between species in A: $p < 0.001$, ANCOVA and B: $p = 0.031$, ANCOVA, and were not significantly different in C. A: *M. arenaria*: $\log MO_2 = -0.847 * \log$ rel. age + 1.83, $r^2 = 0.730$; *L. elliptica*: $MO_2 = -0.28 * \log$ rel. age + 0.99, $r^2 = 0.344$. B: *M. arenaria*:

RCR = -0.0175 * rel. age + 2.996, r^2 = 0.428; *L. elliptica*: RCR = -0.007 rel. age + 2.04, r^2 = 0.221. C: *M. arenaria*: Leak = 0.314 * rel. age + 33.74, r^2 = 0.393; *L. elliptica:* Leak = 0.174 * rel. age + 51.49, r^2 = 0.137.

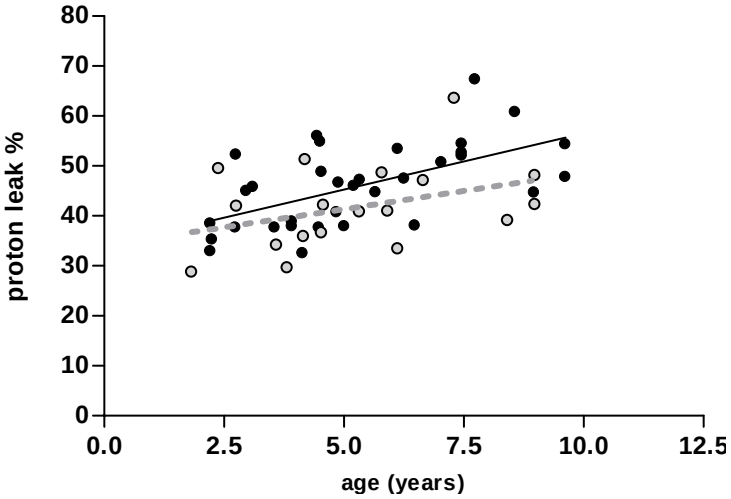

Fig. 8: Proton leak of *M. arenaria* uncorrected (black dots, N = 33) and corrected (grey dots, N = 18) for ROS generation. Regression details and p value for uncorrected values see legend of Fig. 3. For corrected values no significant relationship between age and proton leak was found.

Publication III

Physiological ageing in a polar and a temperate swimming scallop

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Abstract

We compared physiological ageing parameters in two scallops, the temperate *Aequipecten opercularis* and the Antarctic *Adamussium colbecki*. The two species are phylogenetically closely related and display similar lifestyles but have distinctly different maximum lifespans (MLSP). *A. opercularis* does not live longer than 8-10 years, whereas *A. colbecki* lives > 25 years and may even be able to attain > 100 years of age. The development of several physiological ageing parameters over time, chosen according to the “Free radical theory of ageing” (Harman, 1956), was compared in the two species to identify differences in the aging process. In the shorter lived *A. opercularis,* activities of the mitochondrial enzymes cytochrome c oxidase and citrate synthase and of the antioxidant enzyme catalase showed a more pronounced decrease with age than in the longer lived *A. colbecki*. In line with this finding, lipofuscin accumulation increased more distinctly in *A. opercularis* compared to *A. colbecki* while tissue protein content decreased in *A. opercularis* but increased in *A. colbecki*. The better preservation of mitochondrial and antioxidant enzyme activities and the avoidance of waste accumulation may enable *A. colbecki* to live longer than *A. opercularis*. Mitochondrial function investigated in *A. opercularis* showed only minor changes with age and mitochondrial H_2O_2 generation rates were low at all ages. We relate our findings to the “Free Radical - Rate of Living” theory

(Pearl, 1928; Harman, 1956), to the “Uncoupling to Survive” hypothesis (Brand, 2000), and to the particular lifestyle of these scallops.

Keywords:

Ageing; scallops; ROS; mitochondria

1. Introduction

There is much empirical evidence for higher maximum life spans (MLSP) in cold adapted ectotherm species compared to close relatives from temperate waters (Brey, 1991; Brey et al., 1995; Ziuganov et al., 2000; Cailliet et al., 2001; La Mesa and Vacchi, 2001; Philipp et al., 2005 in press-a). We want to explore the physiological principles underlying those differences in MLSP, especially with respect to the accelerating effect of temperature on metabolic rates and reactive oxygen species (ROS) formation.

Two ageing theories link ageing and MLSP to mitochondrial ROS formation: (1) The “Free Radical-Rate of Living” theory (Pearl, 1928; Harman, 1956) predicts a negative correlation between standard metabolic rate (SMR) and MLSP due to increased mitochondrial production of ROS at higher SMR (Ku et al., 1993). In some species, however, this strict relationship between MLSP and SMR is not maintained, especially in primates and birds, which live longer than expected from their SMR (Perez-Campo et al., 1998). (2) The “Uncoupling to Survive” hypothesis by Brand (2000) intends to explain this contradiction by assuming that mitochondrial uncoupling modulates ROS production, thus altering the strict dependency of ROS formation on SMR.

A recent comparison of sessile infaunal soft-shell clams from Antarctica (*Laternula elliptica*) and from temperate regions (*Mya arenaria*) (Philipp et al., 2005 in press-a; Philipp et al., 2005 in press-b), showed that lower metabolic rates of the Antarctic *L. elliptica*, along with the maintenance of a reduced tissue redox state, lower mitochondrial H_2O_2 generation and a less pronounced decline in mitochondrial functions (aerobic capacity, RCR, proton leak) could form the basis of the extended lifespan of the Antarctic species when compared to the temperate *M. arenaria*.

The present study extends our approach to actively swimming bivalves, to see whether there are unifying principles of physiological aging in bivalves or whether an active and energy consuming lifestyle leads to different strategies regarding maintenance of physiological fitness. The Antarctic scallop *Adamussium colbecki* is a common member of Antarctic coastal communities, where it experiences a habitat temperature range of -1.8 to 2.5°C (Berkman, 1990; Berkman et al., 2004). The temperate queen scallop *A. opercularis* is found along the European coast from Norway to the Mediterranean and from the

Adriatic to the west of Ireland, spanning an overall temperature range from 6 to 24°C (Ansell et al., 1991). Both pectinids are epibenthic with a well developed capacity of jet propulsion for swimming (Brand, 1991; Ansell et al., 1998). Despite their close phylogenetic relationship (Canapa et al., 2000) and principal similarity in their lifestyles, the two species differ distinctly in maximum life span (MLSP). A. colbecki is long lived and may reach 100 years and more (Berkman, 1990), whereas MLSP of *A. opercularis*, on the other hand, does not exceed 8-10 years (Ansell et al., 1991).

2. Material and Methods

2.1. Sampling and maintenance

2.1.1. Aequipecten opercularis

Irish Sea *Aequipecten opercularis* were dredged 15 miles south of Port St Mary, Isle of Man in July-August 2003 at about 64 m water depth. Animals were transported to the Port Erin Marine Laboratory and kept in natural sea water flow-through aquaria at ~10°C and 34 PSU for several days prior to experimentation. Experiments with isolated mitochondria were carried out at the Port Erin Marine Laboratory. For all other analyses, samples were freeze clamped immediately and stored in liquid nitrogen for transportation to the Alfred-Wegener-Institute, Bremerhaven.

2.1.2. Adamussium colbecki

Antarctic *Adamussium colbecki* were dredged at Terra Nova Bay (“Road Bay”, 74°43‘ S 164°13’ E) in February 2004. Animals were either sacrificed after collection and the mantle tissue stored in liquid nitrogen, or frozen as a whole in liquid nitrogen. Samples were transported in liquid nitrogen from Antarctica to Genova, Italy and at -80°C from Genova to the Alfred-Wegener-Institute in Bremerhaven.

2.2. Age determination

External annual shell growth checks were used to establish relations between shell height and age in both species. In *A. opercularis* we aged 76 animals and took an additional 200 height-at-age data from Allison (1993). For *A. colbecki* 185 height-at-age data pairs of the Terra Nova population were available from Heilmayer et al. (2003). The von Bertalanffy growth function (VBGF)

$$H_t = H_\infty * (1 - e^{-k * (t - to)})$$

was used to model the relation between shell height and age, where H_t is height at age t, H_∞ is height at infinite age and t_0 is age at which size would be zero, for details see Brey

(2001). To estimate age t from size H_t of our experimental animals, we fitted the inverse VBGF :

$$t = \ln (1 - H_t / H_\infty) / -k + t_0$$

to the height-at-age data sets using the non-linear iterative Newton algorithm.

2.3. Metabolic rate

Standard metabolic rate (SMR) of summer *Aequipecten opercularis* (N = 31, mass range: 219-3411 mg AFDM, size range: 30-80 mm, age range: 1-6 years) was measured at 10°C and 34 PSU in a multi-channel modified intermittent flow system with oxygen microoptodes connected to a Microx TX 2–array (® PreSens GmbH), as previously described in Gatti et al. (2002). Microoptodes were calibrated to 100% oxygen solubility in air-saturated and to 0% in N_2-saturated seawater at experimental temperature. Prior to respiration measurements, individuals of *A. opercularis* were maintained without food during three days, to eliminate effects of specific dynamic action (SDA).

The respiration chambers (200-650 ml) were placed in a plastic tub with filtered seawater with a peristaltic pump (Ismatec) ensuring continuous water circulation. To reduce handling stress, scallops were allowed to accommodate to the respiration chambers over night. After closing the respiration chambers, the decrease in oxygen content from 100% to about 70% oxygen over time was recorded. Individual animals were measured over two to three runs, each, over a time of 2 to 3 hrs. After the measurements, animals were dissected and soft tissue wet mass and dry mass (3 days at 60°C) determined. Tissues were combusted at 500°C over 5 h to determine the ash free dry mass (AFDM). Standard metabolic rates (SMR) were determined after subtraction of the microbial oxygen demand of the system, determined in a parallel blank chamber. Percent O_2 saturation was transformed to micromoles of dissolved oxygen in seawater, using known values of oxygen solubility according to Benson and Krause (1984) and converted to µg O_2. As no living *A. colbecki* individuals were available for SMR measurements, respiration data were taken from Heilmayer and Brey (2003) for *Adamussium colbecki* (N = 85, mass range: 104-3911 mg AFDM, size range: 27-74 mm; age range: 3-10 years) from Terra Nova Bay, measured in austral summer during January/February 2000.

2.4. Mitochondrial measurements

2.4.1. Isolation of mitochondria

Mitochondria were isolated from the mantle tissue of freshly sacrificed bivalves. Tissues of up to three individual *A. opercularis* specimens were pooled for one experiment. About 3 g of mantle tissue were finely chopped in 10 ml ice-cold homogenisation buffer modified

after Moyes et al. (1985) (400 mM sucrose, 70 mM Hepes, 100 mM KCl, 3 mM EDTA, 6 mM EGTA, 1% bovine serum albumine, 1 µl/ml aprotinine, pH 7.3).

Briefly, the tissue was homogenised in a pre-cooled glass/teflon-homogeniser, centrifuged at 1300 g for 15 min at 2°C and the supernatant collected. The pellet was resuspended, homogenised, and again, mitochondria were separated at the same speed. The two supernatants were combined and centrifuged at 10500 g for 10 min to sediment the mitochondria. The resulting mitochondrial pellet was resuspended in 1.5-2 ml assay medium (560 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 70 mM Hepes, 5 mM glutamate, 1 µg/ml aprotinine and 1% bovine serum albumine at pH 7.3).

2.4.2. Respiration and membrane potential of isolated mitochondria

Mitochondrial respiration measurements were carried out at 10°C. Respiration rate was recorded with oxygen microoptodes (TX PreSens GmbH, Neuweiler, Germany) and membrane potential determined using TPMP-sensitive electrodes according to Brand (1995). Oxygen concentrations were calculated using the oxygen solubility ($ßO_2$) according to Johnston et al. (1994) and the atmospheric pressure of the day. 5 mM succinate, with 5µM rotenone to prevent respiration of endogenous NAD-linked substrates (Brand, 1995), were used as substrate and state 3 respiration was induced by addition of 0.15 mM ADP. Non-phosphorylating respiration, oxygen consumption due to proton leakage and ROS formation (state 4+) were recorded after adding 2 µg/ml of the F_0F_1-ATPase inhibitor oligomycin. The percentage of proton leak of state 3 oxygen consumption was calculated as (state 4+/state 3) *100. The respiratory control ratio (RCR), which describes how effectively the respiratory chain is coupled to the ATPase, was calculated according to Estabrook (1967), using state 4+ respiration. The ADP/O ratio was determined by recording the time used for the consumption of the given ADP and the corresponding oxygen consumption. Membrane potential measurements of mitochondria isolated from *A. opercularis* mantle tissue were carried out as described in Keller et al. (2004).

2.4.3. Production of hydrogen peroxide (H_2O_2) by isolated mitochondria

Mitochondrial hydrogen peroxide production was measured fluorimetrically ($\lambda_{exitation}$= 312 nm and $\lambda_{emission}$ = 420 nm) by recording the reaction of H_2O_2 with homovanilic acid (HVA) in the presence of horse radish peroxidase (HRP), using a procedure modified after Miwa et al. (2003). Measurements of the H_2O_2 generation rate of *A. opercularis* mitochondria were performed with a Shimadzu (RF-1501) fluorometer at the Port Erin Marine Laboratory as described in detail in Philipp et al. (2005 in press-b) for *L. elliptica*. Both,

H_2O_2 generation rates and oxygen consumption rates were measured in parallel and related to mitochondrial protein content (for details see Keller et al., 2004).

2.5. Enzyme assays

Enzyme activity was measured in mantle tissue of *A. opercularis* and *A. colbecki*. All samples were measured at 20°C reference temperature. To investigate temperature sensitivity of the different enzymes (Q_{10} values), enzyme activities were also determined at 0-5°C for *A. colbecki* and at 10°C for *A. opercularis*. Data are expressed as international units (µmol of substrate converted to product min^{-1}) per mg protein. Protein content of the extracts was determined by the Biuret method (Kresze, 1988).

2.5.1. Superoxide-dismutase

Superoxide-dismutase was determined after Livingstone et al. (1992). Frozen mantle tissue was ground in liquid nitrogen and homogenised in Tris buffer (20 mM TRIS-HCl, 1 mM EDTA, pH 7.6) 1:8 (w/v). Samples were centrifuged for 3 min at 14000 rpm and at 2°C. SOD activity was measured as degree of inhibition of the reduction of cytochrome c by superoxides generated by a xanthine oxidase/xanthine system at 550 nm in 43 mM potassium buffer with 0.1 mM EDTA, pH 7.8. 1 Unit SOD causes 50% inhibition under assay conditions.

2.5.2. Catalase

Catalase activity was determined after Aebi et al. (1984). Frozen mantle tissue was ground in liquid nitrogen and homogenised in 50 mM phosphate buffer (50 mM KH_2PO_4, 50 mM NA_2HPO_4, pH 7.0) with 0.1% Triton x-100 at 1:5 (w/v). Samples were centrifuged at 13000 g for 15 min at 2°C. The activity was determined by recording the period of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.4 at 240 nm (1 unit).

2.5.3. Cytochrome c oxidase (COX, EC 1.9.3.1) and citrate synthase (CS, EC 4.1.3.7) measurements

Frozen mantle tissue was ground in liquid nitrogen and homogenised with a glass homogeniser (Nalgene, USA) in Tris-HCl buffer (20 mM Tris-HCL, 1 mM EDTA, 0.1% (v/v) Tween® 20, pH 7.4) 1:3 (w/v) for COX, and 1:4 (w/v) for CS.

For COX measurements, homogenates were centrifuged for 10 min at 1000 g and 2°C. COX activity was determined after Moyes et al. (1997) by measuring the oxidation rate of cytochrome c at 550 nm in 20 mM Tris HCL buffer with 0.5% Tween 20, pH 8.0 Activity was calculated using the mmolar extinction coefficient ε_{550}mM 19.1 mM^{-1} cm^{-1} after Hardewig et al. (1999) .

Homogenates for CS activity were sonicated for 15min in a Branson Sonifier 450 (output control 8, Duty cycle 50%) cooled to 0°C and centrifuged at 7400 g for 5 min at 2°C. CS activity was measured after Sidell et al. (1987) recording the absorbance increase of 5 mM DTNB (5,5´dithiobis(2-nitrobenzoic acid)) in 100 mM Tris HCL (pH 8.0), 20 mM acetyl-CoA and 20 mM oxaloacetate at 412 nm. Activity was calculated using the mmolar extinction coefficient ε_{412}mM of 13.61 mM^{-1} cm^{-1}.

2.6. Glutathione content

The concentrations of the oxidised (GSSG) and the reduced form of glutathione (GSH) were measured using high performance liquid chromatography (HPLC) with a method modified after Fariss and Reed (1987) (for a more details see Philipp et al., 2005 in press-a). The measurement is based on the derivatisation of the thiols with dinitrofluobenzene (DNFB). GSH oxidation during extraction is prevented by iodoacetic acid (IAA) binding of GSH. The HPLC system consisted of a NH_2-spherisorb column (240x4 mm, 5µm particles) at 39°C and a binary solvent system of A: 80% methanol/water and B: 80% solvent A and 20% acetate stock (272 g Na-acetate-trihydrate diluted in 122 ml water plus 378 ml glacial acetic acid). Flow rate was 1.2 ml min^{-1} at a maximum backpressure of 2500 psi. The gradient program was: 85% A/ 15% B for 8 min followed by 20 min of linear gradient elution to 45% A/ 55% B and a subsequent 8 min hold. Thereafter the system was returned to the initial conditions within 5 min and re-equilibrated for 15 min.

2.7. Protein oxidation

Detection of protein carbonyl groups as a measure of protein oxidative modifications was carried out after Levine et al. (1990). Samples were homogenised in 50 mM HEPES (125 mM KCl, 0.5 mg/ml leupeptine, 0.7 µg/ml pepstatine, 40 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinine, 1.1 mM EDTA, 0.6 mM $MgSO_4$) and centrifuged at 31000 g for 15 min. Supernatants were incubated for 1 h with 10 mM DNTP (2,4-dinitrophenylhydrazine) in 2 M HCl. Blanks were run without DTNP. After incubation 100% TCA were added to precipitate the protein and centrifuged for 10 min at 11000 g. The protein pellet was washed 3 times with ethanol: ethylacetate (1:1) and resuspended in 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH = 2.3). Samples were measured photometrically using guanidine hydrochloride as a reference. The protein content of the samples was determined by Bradford using bovine serum albumin as a standard.

2.8. Fluorescent age pigment, lipofuscin

Lipofuscin contents were determined by an extraction method modified after Vernet et al. (1988) as described in Philipp et al. (2005 in press-a). The fluorescence intensity of each

sample was determined at an emission maximum of 450 nm for *A. opercularis* and 435 nm for *A. colbecki*. According to Hill and Womersley (1991), lipofuscin concentrations were expressed as relative fluorescent intensity (RFI) using 0.1 µg quinine sulphate per ml 1 N H_2SO_4 as standard.

2.9. Protein content

Protein content was determined as described in Philipp et al. (2005 in press-a). Lyophilised mantle tissue of both species was hydrolysed with 3% NaOH. Samples were centrifuged for 20 min at 5000 rpm to remove cell debris and cooled on ice to facilitate protein precipitation. 60% TCA was added to precipitate the protein. After 20 min at 5000 rpm, the supernatant was discharged and the pellet dissolved in 3% NaOH using 20 ml per initial g tissue dry mass. Protein concentration was determined spectrophotometrically with the Biuret method.

2.10. Statistical analysis

Analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used to analyse the relationship between individual parameters and age and to identify differences between species after testing the data for normality. Lines shown in graphs are regression lines and the upper and lower 95% confidence bands. Differences of Q_{10} values between species were analysed with Students t-test.

3. Results

3.1. Age determination

Relations between shell height and age were well described by the von Bertalanffy growth model in both species (Fig. 1). Age can be inferred from shell height by

age t = ln (1- H_t/108.74)/-0.199 - 1.115, N=276, Mean Squared Error (MSE) = 0.370

in *Aequipecten opercularis* and by

age t = ln (1- H_t/ 122.61)/- 0.083 + 0.237, N=185, MSE = 0.178.

in *Adamussium colbecki*.

3.2. Metabolism

Summer respiration rates (R= µgO_2/Ind/h) of both *A. opercularis* and *A. colbecki* correlated well with body mass M (mg AFDM) (Fig. 2):

A. opercularis (10°C): log(R) = 0.646 * log(M) + 0.447 (N = 31, r^2 = 0.864, p < 0.001)

A. colbecki (0°C): $\log(R) = 0.876 * \log(M) - 0.269$ ($N = 85$, $r^2 = 0.899$, $p < 0.001$)

Slopes and intercepts of the regression lines are significantly different between the two species ($p < 0.001$), but the difference in metabolic rates between the two species decreases with increasing body mass. The 95% confidence range of the predicted values overlap completely beyond 1200 mg body mass, i.e. in *A. opercularis* > 2 years and *A. colbecki*. > 6 years (Fig. 2).

3.3. Age related changes of mitochondrial functions in *A. opercularis*

Oxygen consumption of mantle tissue mitochondria (R_M in nmol O_2 mg protein^{-1} min^{-1}) at *in situ* temperature (10°C) declined significantly with age (Fig. 3):

$R_M = -0.385 * age + 6.460$ ($N = 29$, $r^2 = 0.245$, $p = 0.0063$, age range = 1-5years)

Respiratory control ratio (RCR) and ADP/O ratio declined with age too, but the relationship was much weaker:

$RCR = -0.074 * age + 3.274$ ($N = 34$, $r^2 = 0.111$, $p = 0.054$, age range = 1-5years)

$ADP/O = -0.033 * age + 1.535$ ($N = 33$, $r^2 = 0.118$, $p = 0.05$, age range = 1-5years)

Proton leak showed a slightly positive but non-significant relationship with age ($p = 0.120$).

H_2O_2 generation (nmol H_2O_2 mg protein^{-1} min^{-1}) of isolated mitochondria of *A. opercularis* was extremely low and followed no significant trend with age. Average values across the whole age range (1-5 years) were RCR = 3.070 (S.D. = ±0.312, N = 34), ADP/O = 1.443 (S.D. = ± 0.133, N = 33), Proton leak = 32.84 (S.D. = ± 3.87, N =34), H_2O_2 generation = 0.0025 (S.D. = ± 0.0012, N = 30).

Membrane potential (MP) of isolated mitochondria did not change significantly with age up to 4 years of age (N = 21), but decreased significantly in individuals >4 years (N = 9, Fig. 4):

$MP = -43.24 * age + 330.7$ ($N = 9$, $r^2 = 0.716$, $p = 0.004$)

3.4. Mitochondrial enzyme activities in mantle tissue of ageing *A. opercularis* and *A. colbecki*

Activities of the mitochondrial enzymes citrate synthase (CS) and cytochrome c oxidase (COX) decreased significantly with age in *A. opercularis* but not in *A. colbecki* (Fig. 5A, B). CS values measured at in situ temperatures (data not shown) were not significantly different between both species throughout all ages. At 20°C reference temperature (Fig. 5A) values were in the same range in young individuals of both species. At advanced age (> 5 yrs) *A. colbecki* showed significantly higher values than *A. opercularis*. COX values

were significantly higher in *A. opercularis* compared to *A. colbecki* at in situ and reference temperatures (Fig. 5B).

3.5. Age dependent changes of antioxidant defence mechanisms

Activity of the antioxidant enzyme catalase (U mg protein^{-1}) decreased significantly with age in both species, but with a 5-fold faster decrement in *A. opercularis* compared to *A. colbecki*. (Fig. 6). In young individuals, *A. colbecki* showed lower catalase activities than *A. opercularis*. In old *A. colbecki*, however, activities were in the same range as in old *A. opercularis*. Superoxide-dismutase (SOD) activity (U mg protein^{-1}) was not affected by age in *A. opercularis* (N = 27, p = 0.443, age range = 1-5 years) and was assumed to be independent of age in *A. colbecki*, too (N = 5, age range = 4-8years). Mean (±S.D.) superoxide-dismutase activities at 20°C were significantly lower in *A. colbecki* compared to *A. opercularis* with 10.19 (± 0.89, N = 5) versus 12.77 (± 2.63, N = 27) U mg^{-1} protein (p = 0.040, Student's t-test), respectively.

Q_{10} values of COX, SOD and catalase were not significantly different between species (students t-test), but Q_{10} of CS was significantly lower in *A. colbecki* than in *A. opercularis* with $p < 0.001$ (Table 1).

In both *A. opercularis* and *A. colbecki* glutathione concentrations (GSH, GSSG, tGSH) as well as the ratio of oxidised to reduced glutathione (GSSG/GSH) decreased significantly with age ($p < 0.001$). Slopes did not differ significantly between the two species with respect to each parameter, but intercepts did, i.e. all concentrations were higher in *A. colbecki* compared to *A. opercularis* across the whole age range (Table 2., graph only shown for tGSH and the ratio GSSG/GSH, Fig. 7A and B).

3.6. Age-dependent changes in oxidative damage parameters

Lipofuscin accumulated with age in both species (Fig. 8). Absolute lipofuscin concentrations were in the same range in both species, but accumulation occurred at a 3 times faster rate in *A. opercularis.* Protein carbonyl content, a marker for protein oxidation, did not vary with age in neither species, but was significantly ($p < 0.001$) higher in *A. colbecki* (1.319 nmol carbonyls mg protein^{-1} ± 0.181, N = 24) than in *A. opercularis* (1.016 nmol carbonyls mg protein^{-1} ± 0.072, N = 24).

3.7. Age-related changes in protein content in mantle tissue

Mantle tissue protein content showed opposing trends with age in both species (Fig. 9). In *A. opercularis*, protein concentrations decreased significantly from around 309 mg to 267 mg protein g^{-1} dry mass between 1 and 5 years of age, whereas in *A. colbecki* values increased from around 193 to 290 mg protein g^{-1} dry mass between animals aged 4 and 16 years.

4. Discussion

Metabolic Rate

According to the predictions of the “Free Radical - Rate of Living” theory (Pearl, 1928; Harman, 1956) low standard metabolic rates (SMR) of marine ectotherms living at low temperatures might be one reason for higher maximum lifespans in these animals compared to their temperate counterparts. Recent studies of pectinid SMR are in line with this hypothesis and revealed a positive relation of metabolic rates and ambient temperature across a wide latitudinal gradient (Heilmayer and Brey, 2003; Heilmayer et al., 2004). In our study whole animal specific metabolic rates of Antarctic and temperate scallop species were lower in small/young Antarctic *A. colbecki* individuals compared to temperate *A. opercularis* at the same body size (200-500 mg AFDM), which is in line with the pattern elaborated by Heilmayer et al. This difference, however, diminished with increasing body mass and became negligible above 1200 mg AFDM, corresponding to individual ages of > 2 years in *A. opercularis* and > 6 years in *A. colbecki* (Fig. 2). In larger animals, the similarity of metabolic rates despite the about 10°C difference in habitat temperature clearly indicates that the metabolic rate of *Adamussium colbecki* is fully cold compensated when compared to the SMR of *A. opercularis*. These findings may be related to the fact that we measured only summer metabolic rates. Summer rates, however, can be affected heavily by somatic growth, gonad development and environmental parameters such as food availability and temperature elevation (Peck et al., 1987; Shumway et al., 1988; Peck, 1998; Brockington, 2001; Lesser and Kruse, 2004). These processes are likely cold compensated in the Antarctic scallops, causing elevated metabolic rates in the adults and, possibly, the steeper slope of the allometric metabolic rate-mass relationship. The “true” difference in baseline metabolic rates may accordingly be obscured. Year round measurements are necessary to obtain a realistic estimate of metabolic rates which takes such effects into account (Clarke, 1988). However, even if a difference of metabolic rates between both scallop species seems likely, it has not been documented. Hence the question arises, if other parameters like mitochondrial ageing, antioxidant defence and oxidative damage are more distinctly different, and whether these parameters show different trends with age in both species, which may explain the higher MLSP of the polar compared and the temperate scallop.

Mitochondrial ageing

Age related changes in mitochondrial function (respiration, RCR, ADP/O, proton leak and membrane potential) in mantle tissue of *A. opercularis* were small (Fig. 3), but followed a pattern already known from humans (Trounce et al., 1989; Cooper et al., 1992; Boffoli et

al., 1994), other terrestrial vertebrates (Goodell and Cortopassi, 1998; Kokoszka et al., 2001; Hagen et al., 2002; Lopez-Torres et al., 2002; Ventura et al., 2002; Grattagliano et al., 2004), and invertebrates (Sohal et al., 1995) including marine bivalves (Philipp et al., 2005 in press-b). Protein specific activities of the mitochondrial enzymes citrate synthase (CS) and cytochrome c oxidase (COX) declined significantly with age in *A. opercularis*, as found in human, rat and mouse (Paradies et al., 1993; Rooyackers et al., 1996; Paradies et al., 1997; Kokoszka et al., 2001). This change is held responsible for the decline in aerobic capacity and muscle performance associated with age in most organisms (Rooyackers et al., 1996). In contrast to *A. opercularis*, mitochondrial enzyme activities were independent of age in *A. colbecki* (Fig. 5). The relevance of stable COX and CS activities for delayed mitochondrial senescence remains questionable, as mitochondrial functions could not be measured directly in *A. colbecki.* In the infaunal soft-shell clams *M. arenaria* (MLSP 13 years) and *L. elliptica* (MLSP 36 years) COX and CS activities were stable but the decline in mitochondrial functions in *M. arenaria* was even more pronounced than in *A. opercularis*. Regarding energy conservation, however, stable CS and COX activities in *A. colbecki* might play a role in life span extension as they indicate better preservation of tissue aerobic capacity with age.

The very minor age-related decline in mitochondrial functioning of *A. opercularis* came as a complete surprise, as we would have expected a more rigid onset of mitochondrial deterioration in this short lived and actively swimming species, especially when compared to the sessile clams. Moreover, H_2O_2 generation rates with either succinate plus rotenone, or pyruvate and malate (without rotenone), or at higher assay temperatures (20°C) were close to zero in *A. opercularis*, despite intense mitochondrial respiration and a low proton leak (33%). In contrast, the longer lived temperate soft shell clam *M. arenaria* showed far higher H_2O_2 generation rates per mg mitochondrial protein than *A. opercularis*, despite lower state 3 respiration rates (individuals > 5years) and a generally higher mitochondrial proton leak (Philipp et al., 2005 in press-b).

The lower proton leak of *A. opercularis* mitochondria compared to mitochondria of the less active infaunal bivalves *M. arenaria* and *L. elliptica* (Philipp et al., 2005 in press-b), corresponds to the more active lifestyle of the scallop. Following Brand's (2000) rationale of mild uncoupling, less proton leakage would, however, cause higher rather than lower ROS generation. This raises the question whether some special mechanism may prevent ROS generation in mitochondria of *A. opercularis* or even of scallops in general. Preliminary data point into this direction, as H_2O_2 generation rates (at 0°C) of mitochondria isolated from three available *A. colbecki* individuals were below detection limits, whereas

in the polar mud clam *L. elliptica* H_2O_2 generation of isolated mitochondria was well detectable at 0°C (Philipp et al., 2005 in press-b).

High glutathione concentrations were found in the tissue of *A. opercularis* and *A. colbecki,* but GSSG concentrations and tissue redox states (GSSG:GSH) were lower in the scallops than in polar and temperate mud clams (Philipp et al., 2005 in press-a). This indicates lower ROS scavenging activity of the glutathione system in scallops and would coincide with lower rates of mitochondrial ROS production. Moreover, comparable or lower catalase and superoxide dismutase activity levels in scallops than in mud clams indicate that the more active scallop lifestyle does not imply vastly higher mitochondrial ROS formation.

In both scallop species the concentration of tissue glutathione decreased linearly with age (Fig. 7a, Table 2), which is in line with findings in humans, rats, insects and marine bivalves (Sohal et al., 1987; Sanz et al., 1996; Canesi and Viarengo, 1997; Hernanz et al., 2000; Philipp et al., 2005 in press-a). Likewise, the ratio of GSSG:GSH decreased with age in both species (Fig. 7b). Thus, we suggest that the scallops adjust the glutathione content to match the age-related decline of mass specific respiration, as reported for *Mytilus edulis* (Sukhotin and Pörtner, 2001). As discussed for the polar clam *L. elliptica* where a similar development of GSH concentration and tissue redox ratio was found, the glutathione level should suffice to maintain low tissue oxidation in aged animals (Philipp et al., 2005 in press-a).

In contrast to the uniform decrease in glutathione, the decrease in catalase activity was much more pronounced in *A. opercularis* compared to *A. colbecki*, indicating a more rapid loss of antioxidant capacity with age in the temperate scallop. Accordingly, the increase in lipofuscin concentrations was more explicit in the temperate scallop, indicating faster oxidation of lipids and proteins in *A. opercularis* with age, as antioxidative capacity declined.

The comparatively slow increase in lipofuscin levels (Fig. 8) and the preservation of mantle protein content (Fig. 9) with age in *A. colbecki* support the idea that avoidance of waste accumulation and maintenance of cellular integrity are prerequisites of longevity in the polar scallop. The higher protein carbonyl content in *A. colbecki* compared to *A. opercularis* might therefore not necessarily reflect higher protein oxidation rates, but may result from thermally reduced proteolytic activity in *A. colbecki* and thus higher steady state levels of oxidized protein at 0°C ambient water temperature. Further studies are necessary to elucidate the underlying mechanisms.

The importance of a high level of physiological performance in active animals

Mitochondria from mantle tissue of the temperate scallop *A. opercularis* exhibit a slower senescence than mitochondria from the temperate mud clam *M. arenaria*, most likely owing to a suppression of mitochondrial ROS production. COX and CS activities, however, decrease with age in *A. opercularis*, while remaining constant in *M. arenaria*. This could indicate an age related decrease in mitochondrial numbers in mantle tissue in *A. opercularis* and, therefore, a loss in tissue aerobic capacity with age. So far age related changes in mitochondrial numbers have only been reported for human liver, where mitochondrial number per cell decreases with age (Tauchi and Sato, 1968).

A prerequisite for an active lifestyle is the maintenance of cellular functions and fitness on a high level to insure maximal scope for activity. *A. opercularis* is an active swimmer with fast escape reactions when disturbed (see e.g. Chapman, 1981). The Antarctic *A. colbecki* features the same active lifestyle to escape scouring icebergs and predators like the fish *Trematomus bernacchii* and invertebrates (*Neobuccinum eatoni, Paraborlasia corrugata*) (Ansell et al., 1998; Vacchi et al., 2000; Peck et al., 2004). A comparison of mature and immature scallops *Chlamys islandica* showed that reduced CS activity and mitochondrial capacity caused a decrease of adductor muscle aerobic capacity in mature specimens and subsequently a delay of recovery after exhaustive swimming, which requires aerobic metabolism (Livingstone et al., 1981; Brokordt et al., 2000). Hence, a decrease in mitochondrial capacity and the decline in COX and CS activity with age, as found in *A. opercularis* may significantly reduce fitness. Corresponding observations were reported by Brand (1991), who found a higher threshold stimulus to elicit swimming and less frequent swimming in larger (older) *A. opercularis*. This, in turn, could cause an increase of extrinsic mortality (= elevated susceptibility to predation) and thus a comparatively shorter MLSP in such highly active animals. On an evolutionary scale, these animals may have adjusted their life history and physiological aging strategies for an optimal functioning until reaching a threshold, where maintenance of physiological competitive capacities requires more energy than justified by the reproductive gain. A theoretical study by William and Day (2003) presented the rationale that an increase in interactive extrinsic sources of mortality (e.g. predation) may select for slower senescence deterioration early in life. A recent study by Reznick et al. (2004) on guppies indeed indicates such a relation between predation pressure and senescence. Although scientific proof for the validity of this concept for *A. opercularis* has still to be put forward, these ideas offer an explanation for the combination of short MLSP and minor decrements of mitochondrial functions with age in *A. opercularis*. In the Antarctic *A. colbecki*, CS and COX activities even remained stable for up to 16 years of age, hence the loss of fitness

and the corresponding increase of extrinsic mortality may start at higher age. Animals across the full age range (MLSP ~45 years), however, have to be studied to see whether physiological fitness in *A. colbecki* is also preserved until close to MSLP as seen in *A. opercularis.*

Conclusions

Summer standard metabolic rates of young Antarctic *A. colbecki* were lower than in young temperate *A. opercularis,* whereas in older individuals no difference was found between both species. Annual metabolic rates of *A. colbecki* however might be generally lower than in *A. opercularis* when taking into account the difference in timescales of winter and summer periods in the Antarctic and temperate environment.

A less pronounced decrease in mitochondrial (COX, CS) and antioxidant enzyme activities (catalase) as well as a less pronounced increase in lipofuscin accumulation with age was found in *A. colbecki* compared to *A. opercularis* which indicate a slower physiological ageing in the polar scallop, enabling *A. colbecki* to reach a higher MLSP than *A. opercularis*. Interestingly, H_2O_2 generation rates were extremely low in *A. opercularis* and below detection limit in *A. colbecki*. The scallops differ distinctly from temperate and Antarctic mud clams (Philipp et al., 2005 in press-b), in which especially the temperate mud clam *M. arenaria* showed higher H_2O_2 generation rates than *A. opercularis* despite higher MSLP. Changes in mitochondrial functions were less pronounced in *A. opercularis* than in *M. arenaria*. We hypothesise that *A. opercularis* and *A. colbecki* keep mitochondrial performance on a high level throughout all ages to retain the active (swimming) lifestyle and to keep extrinsic mortality low. Compared to *M. arenaria*, the marginal decrease in mitochondrial function with age in *A. opercularis* might already be sufficient to enhance extrinsic mortality and contribute to the short MLSP. The minor decrease in mitochondrial functions with age may indicate a slower senescence of *A. opercularis* mitochondria compared to mitochondria from *M. arenaria*. Nonetheless, the pronounced decline in CS and COX activities with age in *A. opercularis*, while activities remained constant in *M. arenaria*, could indicate a decrease in tissue aerobic capacity which would finally lead to a loss in scope for activity and subsequently, to increased extrinsic mortality.

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Tables

Table 1: Q10 values of mitochondrial, cytochrome c oxidase (COX) and citrate synthase (CS) and antioxidant, superoxide dismutase (SOD and catalase, enzymes of *A. colbecki* and *A. opercularis*.

	A. colbecki	*A. opercularis*
COX	1.10 ± 0.51 (N = 8)	1.65 ± 0.74 (N = 27)
CS*	1.54 ± 0.11 (N = 15)	1.74 ± 0.15 (N = 30)
SOD	1.07 ± 0.26 (N = 7)	1.07 ± 0.27 (N = 25)
catalase	1.37 ± 0.09 (N = 17)	1.54 ± 0.38 (N = 25)

*= significantly different between species with $p < 0.001$.

Table 2: Regression equations of whole (tGSH), reduced (GSH) and oxidised (GSSG) glutathione in nmol per g wet mass and the ration of reduced to oxidised (GSSG:GSH) glutathione vs. age in years in mantle tissue of *A. opercularis* and *A. colbecki*.

Species	Equation	N	r^2	P	Age range (years)
A. opercularis	tGSH = -94.74 * age + 904.1	28	0.727	< 0.001	1-5
	GSH = -76.56 * age + 788.0	28	0.664	< 0.001	1-5
	GSSG = -9.092 * age + 58.08	28	0.631	< 0.001	1-5
	GSSG:GSH = -0.0098 * age + 0.0824	28	0.399	< 0.001	1-5
A. colbecki	tGSH = -91.82 * age + 1951	26	0.676	< 0.001	3-14
	GSH = -60.56 * age + 1448	26	0.653	< 0.001	3-14
	GSSG = -14.61 * age + 240.0	26	0.651	< 0.001	3-14
	*GSSG:GSH = -0.0081 * age + 0.1861*	*26*	*0.524*	*< 0.001*	3-14

Figures

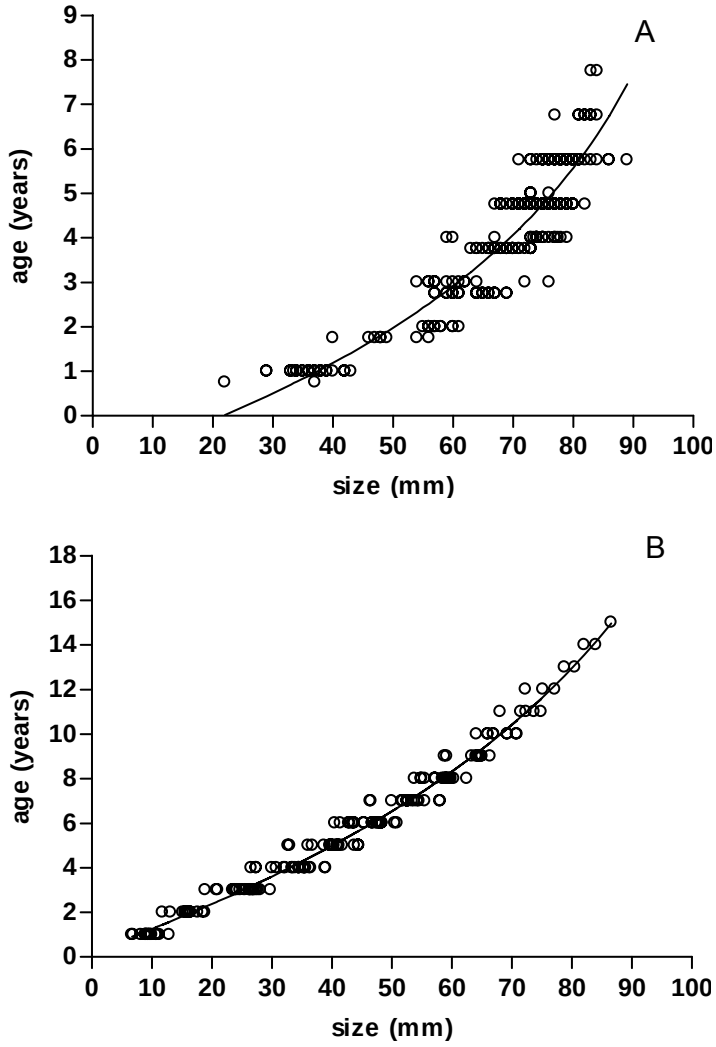

Fig. 1: Inverse Von Bertalanffy growth model of A: *Aequipecten opercularis* from the Isle of Man, age t = ln (1- H_t/108.74)/-0.199 - 1.115, N = 276, Mean Squared Error (MSE) = 0.370 and B: *Adamussium colbecki* from Terra Nova Bay age t = ln (1- H_t/ 122.61)/- 0.083 + 0.237, N=185, MSE = 0.178. The corresponding models to estimate size from age are: $H_t = 84.47 * (1 - e^{-0.427 * (age + 0.347)})$, N = 276, MSE = 15.569 for *A. opercularis*, and $H_t = 126.30 * (1-e^{-0.078 * (t-0.103)})$, N = 185, MSE = 7.189 for *A. colbecki*.

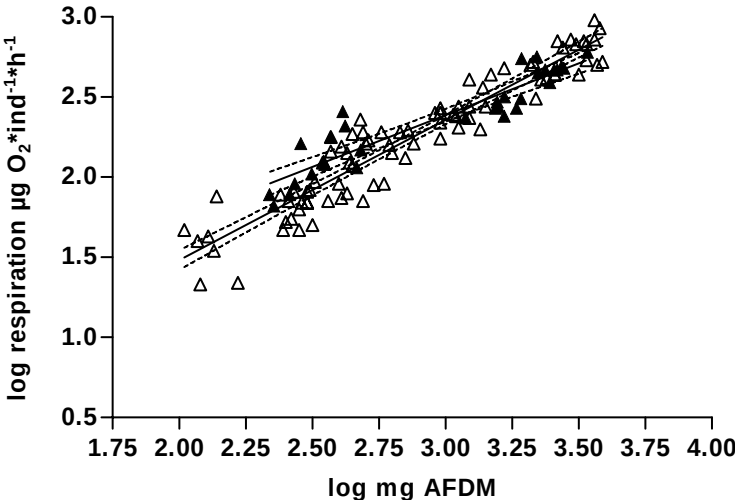

Fig. 2: Standard Metabolic Rate. Summer Respiration rates of *A. opercularis* at 10°C (filled triangles) and of *A. colbecki* at 0°C (open triangles, Heilmayer and Brey, (2003) measured at 0°C. *A. opercularis*: log(R) = 0.646 * log(AFDM) + 0.447; r^2 = 0.864, p < 0,001. N = 31, age range: 1-6 years; *A. colbecki*: log(R) = 0.876 * log(AFDM) – 0.269; r^2 = 0.899, p < 0,001. N = 85, age range: 3-10 years.

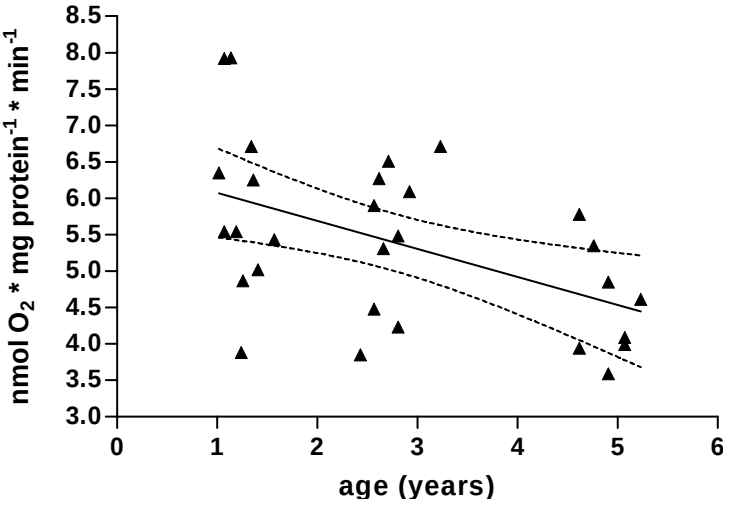

Fig. 3: Decline in state 3 respiration of isolated mitochondria from *A. opercularis* mantle tissue at mean *in situ* temperatures (10°C) with age. Triangles represents between 1 and 3 replicate measurements per mitochondrial isolation. R_M = -0.385 * age + 6.46, N = 29, r^2 = 0.245, p = 0.0063, age range = 1-5 years.

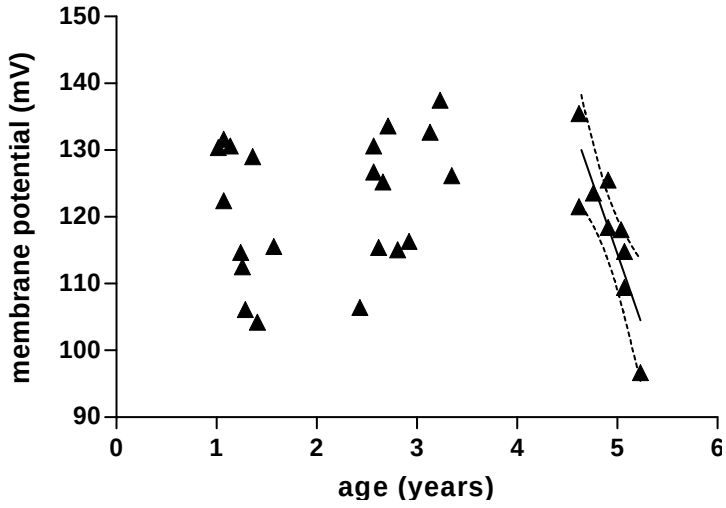

Fig. 4: Membrane potential (MP) of isolated mitochondria from *A. opercularis* mantle tissue at mean *in situ* temperatures (10°C) versus age. Each triangle represents the mean of up to 3 replicate measurements, N = 30. No significant relationship between MP and age of individuals < 4years (N = 21, p= 0.293), but in individuals > 4years with MP = -43.24 * age + 330.7, N = 9, r^2 = 0.716, p = 0.004.

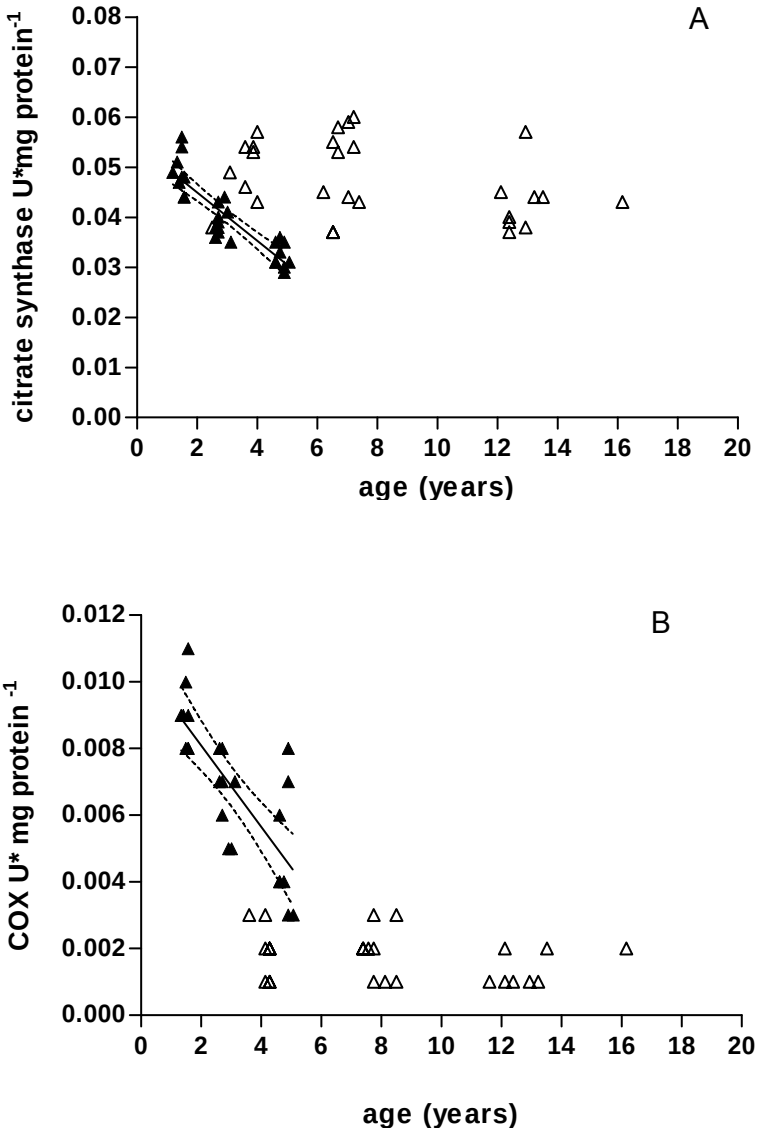

Fig. 5: Activities of (A) citrate synthase (CS) and (B) cytochrome c oxidase (COX) at 20°C in mantle tissue of *A. opercularis* (filled triangles) and *A. colbecki* (open triangles) versus age. Data represent means of 2-3 replicate measurements. Slopes differed significantly between species ($p < 0.001$, ANCOVA).

CS: *A. opercularis*: CS = -0.0048 * age + 0.055, N = 28, $r^2 = 0.786$, $p < 0{,}001$, age range = 1-5years; *A. colbecki*: no significant relationship, N = 28, age range = 2-16 years.

COX: *A. opercularis*: COX = -0.00122 * age + 0.010, N = 24, $r^2 = 0.601$, $p < 0{,}001$, age range = 1-5 years; *A. colbecki*: no significant relationship, N = 26, age range = 2-16 years.

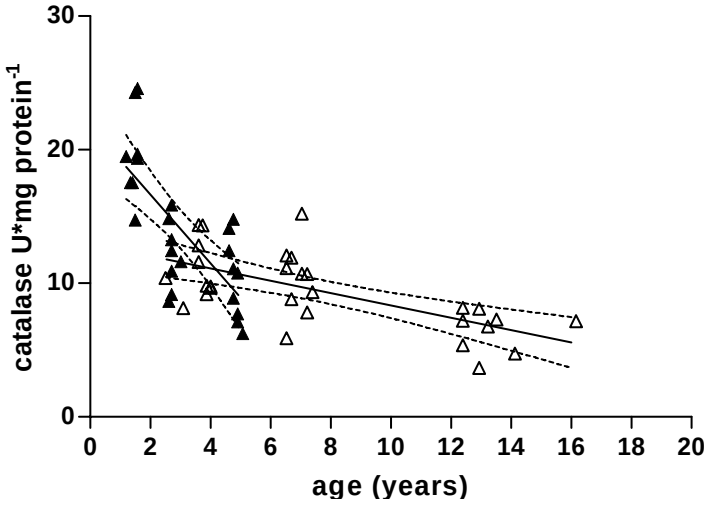

Fig. 6: Activities of catalase at 20°C in mantle tissue of *A. opercularis* (filled triangles) and *A. colbecki* (open triangles) versus age. Each symbol represents the mean of 2-3 replicate measurements. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *A. opercularis*: catalase activity = -2.564 * age + 21.75, N = 25, $r^2 = 0.540$, $p < 0.001$, age range: 1-5 years; *A. colbecki*: catalase activity = -0.463 * age + 12.97, N = 29, $r^2 = 0.438$, $p < 0.001$, age range: 2-16 years.

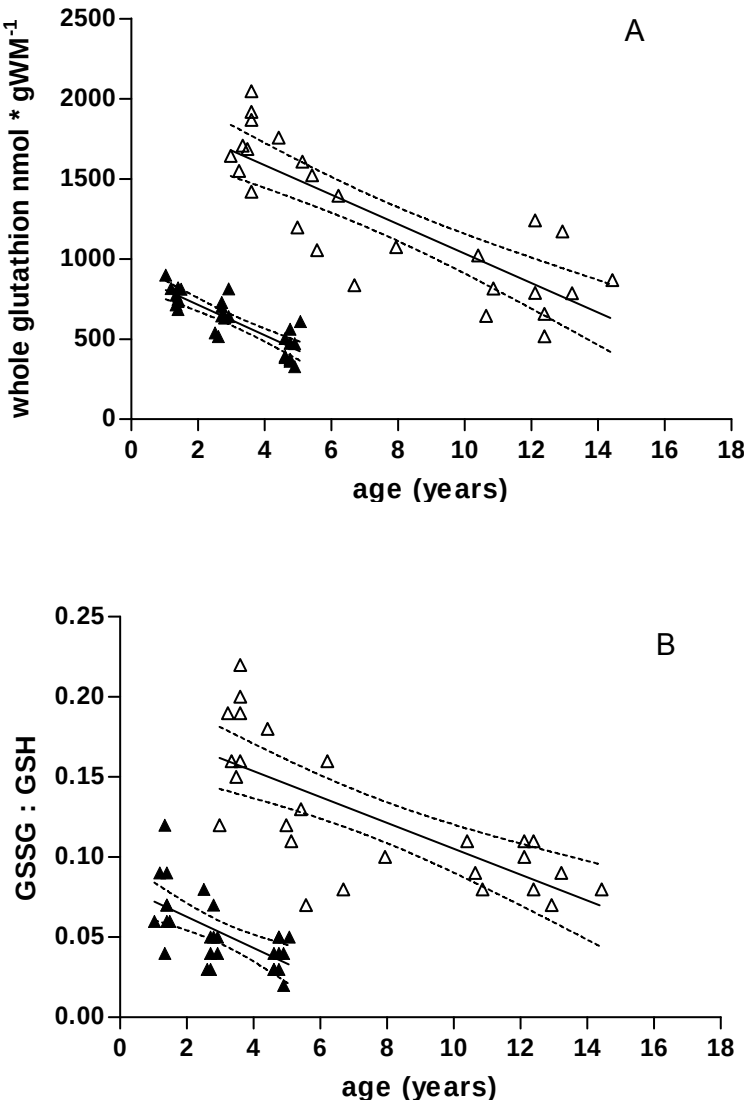

Fig. 7: Decrease of (A) total (tGSH = 2 * GSSG+GSH) and (B) the ratio of oxidised to reduced glutathione (GSSG:GSH) in mantle tissue of *A. opercularis* (filled triangles, N = 28) and *A. colbecki* (open triangles, N = 26) with age. Each symbol represents the mean of duplicate measurements of 1 or 3- 4 pooled animals. Slopes were not significantly different between species. *A. opercularis*: tGSH = -94.74 * age + 904.1, r^2 = 0.727; GSSG:GSH= -0.0098 * age + 0.0824, r^2 = 0.399. *A. colbecki*: tGSH = -91.82 * age + 1951, r^2 = 0.676; GSSG:GSH = -0.0081 * age + 0.1861, r^2 = 0.524. ($p > 0.001$ for all equations).

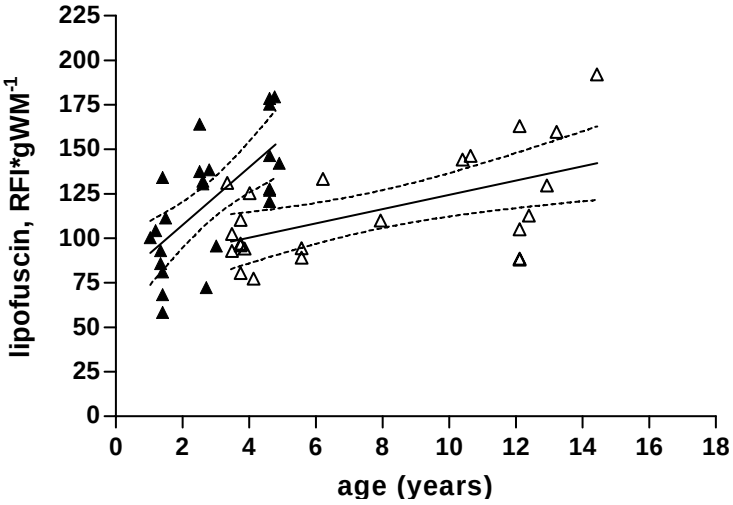

Fig. 8: Lipofuscin content versus age in mantle tissue of *A. opercularis* (filled triangles) and *A. colbecki* (open triangles). Units of lipofuscin content are relative fluorescence intensity (RFI) in the mantle tissue. Slopes differed significantly between species (p = 0.003, ANCOVA). *A. opercularis*: lipofuscin = 16.24 * age + 74.88; N = 24, r^2= 0.453, p < 0.001, age range: 1-5 years; *A. colbecki*: lipofuscin = 4.009 * age + 84.27; N = 24, r^2 = 0.312; p = 0.005, age range: 3-14 years.

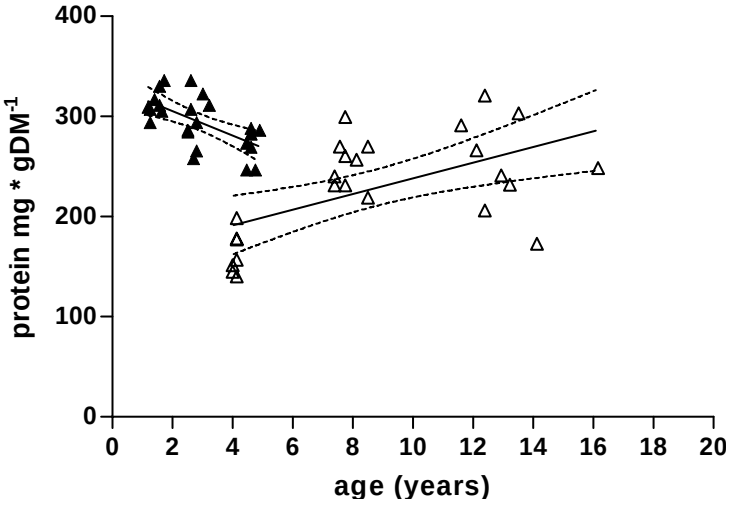

Fig. 9: Protein content (mg protein g^{-1} dry mass) in mantle tissue of *A. opercularis* (filled triangles, N = 25, age range: 1-5 years) and *A. colbecki* (open triangles, N = 25, age range: 4-16 years) plotted against chronological age (years). Data represent means of duplicate measurements. Slopes differed significantly between species (p < 0.001, ANCOVA). *A. opercularis*: protein content = -12.17 * age + 329.7, N = 25, r^2 = 0.396, p < 0.001; *A. colbecki*: protein content = 7.821 * age + 160.1, N = 25, r^2 = 0.324, p = 0.003.

4. DISCUSSION

In the following section I will discuss the investigated ageing parameters of all 4 bivalve species in the context of existing literature about ageing patterns in marine ectotherms as well as in terrestrial vertebrates and invertebrates.

4.1. Chronological age determination and Von Bertalanffy Growth models (VBGF)

For the investigation of physiological ageing patterns in bivalves, taken from the natural environment, the ability to determine the age of individual bivalves was a prerequisite. Hence Von Bertalanffy growth models (VBGF) were fitted to size-at-age data of each investigated species population (Publ. I and III). Individual age could thus be determined from the shell size of an animal. Regarding the two scallop species and *M. arenaria,* the inverse models were sufficient accurate to estimate age from shell size of the experimental specimens (Publ. I and III). In *L. elliptica*, however, the high variability of the size-to-age relationship did not allow for a reliable age-from-size estimates (Fig. 4.1.). Hence, each animal was aged individually (Publ. I).

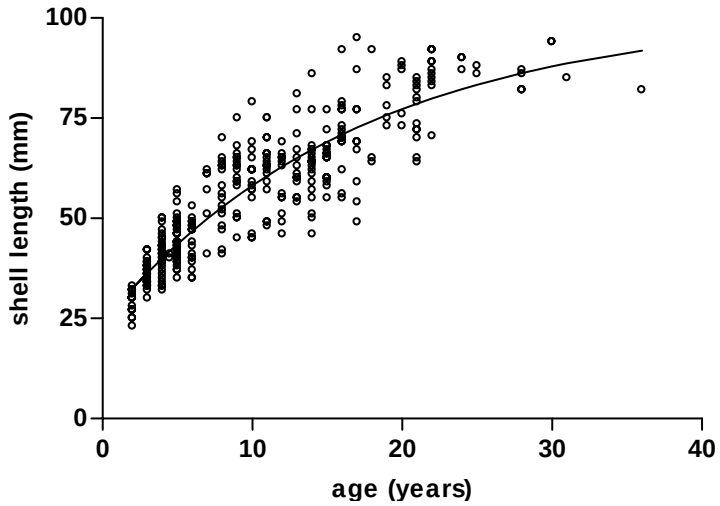

Fig. 4.1.: Length-at-age data points of 347 individually aged *L. elliptica* shells and the corresponding Von Bertalanffy growth model (black line). $S_t = 101.39 * (1-e^{-0.060 * (t + 0.00)})$ N = 347, $r^2 = 0.806$.

The importance of a specific VBGF for each investigated population becomes clear after having a look at existing VBGFs in the literature for the different species. Published data of *M. arenaria* indicate that the bivalves growth is heavily affected by environmental conditions such as temperature (Matthiessen, 1960; Appeldoorn, 1983), currents and sediments (Newell and Hidu, 1982; Brousseau and Baglivo, 1987; Grant and Thorpe,

1991), or tidal regime (Roseberry et al., 1989). Hence, growth function may be highly site specific, as shown in populations of *M. arenaria* from the East and West Coast North-America and the Atlantic east coast of Northern Europe (Fig. 4.2).

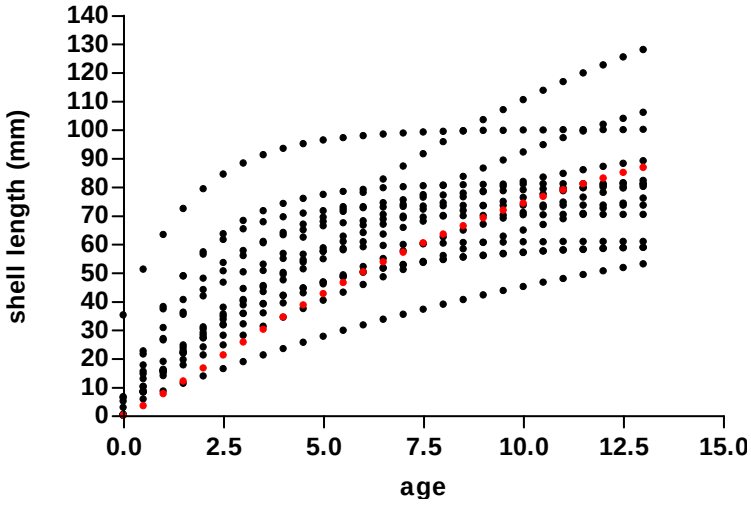

Fig. 4.2: VBGM of *M. arenaria* populations of East and West Coast North-America and the Atlantic east coast of Northern Europe (Netherlands to Norway). The VBGF of the present study is marked with red dots. VBGF from: Brousseau and Baglivo (1987), Palacios et al. (2000), Winther and Gray (1985), Warwick and Price (1976), Munch-Petersen (1973) and Brousseau (1979).

Also the technique used for age determination might cause some variation, as age rings in *M. arenaria* are not always clearly visible (Newcombe, 1936). In the present study age rings were distinguished from disturbance rings by isotope analysis and additionally, age rings were visible on the outer shell surface and x-ray photographs (Fig. 4.3). As some shells, especially from old animals, did not show clearly defined rings on the shell surface, possibly due to abrasion, the resulting length-at-age curve could be used to determine the age of even those shells lacking defined rings.

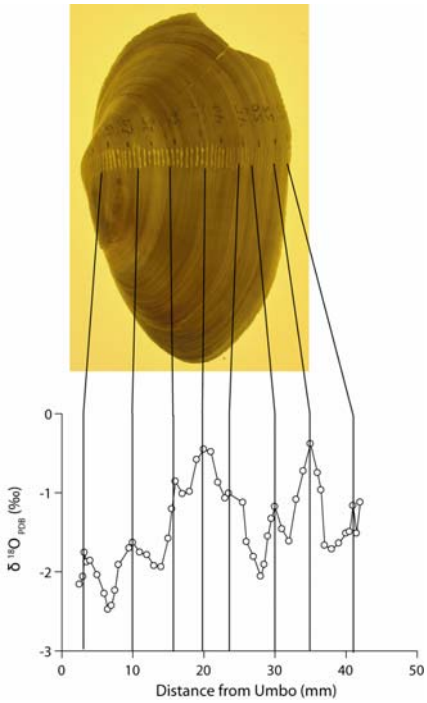

Fig.4.3: *M. arenaria* shell and corresponding $\delta^{18}O$ stable oxygen isotope profile. Annual growth checks coincide with $\delta^{18}O$ peaks, i.e. temperature minima.

The VBGF established for *A. opercularis* is in line with a model established by Heilmayer et al. (2004b) for an *A. opercularis* population of the Western English Channel (Fig. 4.4). The English Channel population showed a similar growth rate in young *A. opercularis* but a lower infinite height (H_∞) than the population from the Isle of Man. Again the importance of specific growth models for individual age

determination becomes clear. Growth models obtained for *A. colbecki* are reviewed and discussed in detail by Heilmayer et al. (2003).

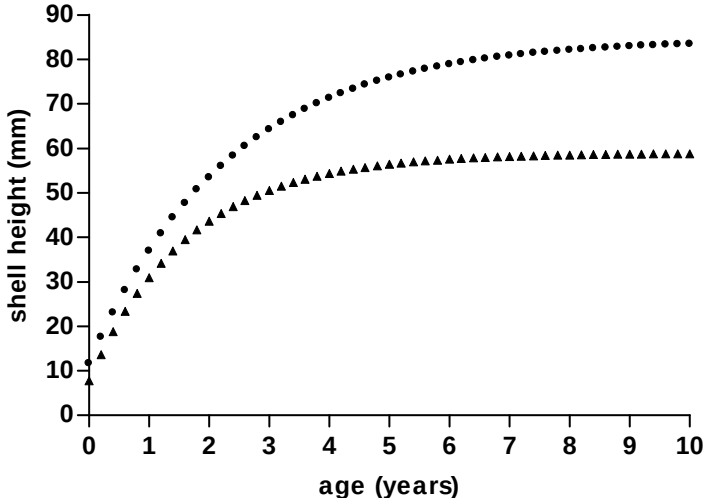

Fig. 4.4. Von Bertalanffy growth models of different *A. opercularis* populations. *A. opercularis* Isle of Man (15 miles south of Port St Mary): filled circles = this study and Allison (1993). *A. opercularis* western English Channel: filled triangles = Heilmayer et al. (2004).

4.2. Separating age effects from size effects

Numerous invertebrate species, including marine bivalves, continue to grow throughout their whole life and therefore the increase in chronological age is always accompanied by an increase in shell size and body mass. The distinction of mere age effects from size effects on physiological parameters is therefore a problem in ageing studies of marine bivalves. Alexey Sukhotin for example investigated ageing parameters in 3 size groups (small, medium and large) of *Mytilus edulis,* with each size group showing a similar age range. He found catalase activity correlated with size but not with age whereas lipofuscin content was exponentially correlated with age but not with size. Standard metabolic rate (SMR) and citrate synthase activity were correlated with both, age and size (Sukhotin and Pörtner, 2001; Sukhotin et al., 2002; Sukhotin et al., 2003). Other parameters like superoxide dismutase activity or malondialdehyde concentration did not show any correlation with either age or size.

In the present study, age of each *L. elliptica* individual was determined directly, and therefore the relation of age and body mass (i.e. size) to physiological ageing parameters could be evaluated to some extent (Table 2 in Publ. I). The strong intercorrelation between age and size (mass), however, makes the proper separation of age effects and size effects impossible. Consequently, physiological parameters were related to age and

size in quite similar ways. Age effects and mass effects, however, were always slightly different as well as of slightly different significance. Age effects on physiological parameters appeared always to be a bit stronger (higher F value in the ANOVA) than size effects, especially on lipofuscin i.e., the patterns observed here might indeed reflect the aging process and not only body growth. In *M. arenaria*, *A. opercularis* and *A. colbecki* such a differentiation was not attainable since the experimental animals were not aged directly, but indirectly from size by the inverse VBGF. To circumvent this problem in future studies, the investigation of ageing parameters in differentially aged individuals of similar size could be useful e.g. in individuals which have already reached the upper, flat part of the VBGF where growth has nearly come to a stop.

4.3. Standard metabolic rate in polar and temperate bivalves

According to the “Free Radical-Rate of Living” theory (Pearl, 1928; Harman, 1956) lower SMR and therefore, lower generation rate of reactive oxygen species, could be a prime factor responsible for a longer MLSP. Studies favouring this theory were carried out with different ectothermal species (insects), where a reduction of metabolic rate by a decrease in temperature or decreased levels of physical activity, resulted in an increase in MLSP (Miquel et al., 1976; Sestini et al., 1991; Sohal, 1991). The comparison of the SMRs obtained in the temperate and polar mud clams of the present study are in line with this hypothesis, as the short-lived *M. arenaria* (MLSP 13 years) showed a 2-fold higher SMR at the same AFDM than the longer-lived *L. elliptica* (MLSP 36 years), at mean environmental temperature (Fig. 2 in Publ. I). The low SMR of *L. elliptica* compared to *M. arenaria* might therefore be one factor contributing to a higher MLSP of the polar mud clam. Calculating life-long aerobic expenses for the maximum age of 36 years, a *L. elliptica* individual showed a 3-fold higher energy turnover than a *M. arenaria* individual within 13 years (MLSP). This demonstrates that, indeed, the rate of respiration and not the accumulated lifetime aerobic energy turnover determines MLSP.

The comparison of an Antarctic (*Adamussium colbecki*) and a temperate (*Aequipecten opercularis*) scallop species, however, did not yield such a straight forward picture (Fig. 2 in Publ. III). While young individuals of both species show distinct SMRs, with a lower SMR in Antarctic compared to temperate individuals at equal body mass, no significant difference was found between *A. opercularis* and *A. colbecki* individuals over 2 and 6 year old, when compared at their habitat temperature. As already outlined in publication III, mean annual SMR might nevertheless be lower in *A. colbecki* than in *A. opercularis* when taking differences in the seasonality of food supply and in temperature between the Antarctic and temperate environment into account. This assumption is corroborated by a study of Heilmayer et al. (2004a) who analysed compiled data sets of metabolic rates of

13 different pectinid species (82 studies) and found a decrease in metabolic rate with decreasing environmental temperature.

However, the difference in SMR between both scallop species is speculative and could not be experimentally confirmed. Therefore, the higher MLSP of the polar compared to the temperate scallop cannot be explained by the “Free Radical-Rate of Living“ theory if only based on the comparison of SMR alone.

Since SMRs obtained for the Antarctic and temperate scallops did not show a significant difference during the major part of their lives, above 2 years of age in *A. opercularis* and above 6 years in *A. colbecki*, *A. colbecki* must display higher life-long aerobic expenses at a maximum age of ~45 years than *A. opercularis* at 10 years. This difference is even larger between the two scallops than between the two clam species. In a comparative study of two rodent species, a higher “metabolic potential“, which can be compared to the lifetime aerobic expenses calculated in the present study, was also found in the long-lived than in the short-lived species (Sohal et al., 1993). The study revealed that both rodents had similar SMRs but the longer-lived species had lower rates of mitochondrial ROS generation and higher antioxidant capacities compared to the shorter-lived species, resulting in lower accumulation of oxidative damage. This was held responsible for the longer MLSP.

As already outlined in the introduction, a higher SMR does not necessarily entail higher ROS generation as previously assumed by Fleming et al. (1981). Especially in ectotherms from cold environments, or experiencing seasonal cold, higher cristae densities and altered membrane fatty acid composition of cold adapted mitochondria can be found (Archer and Johnston, 1991; Johnston et al., 1994; St.-Pierre et al., 1998; Sommer and Pörtner, 2002), which can modulate free radical leakage from mitochondria and thus the simple relationship between SMR and ROS generation and therefore between SMR and MLSP. Moreover, higher antioxidant capacities were found in polar compared to temperate ectotherms (Abele and Puntarulo, 2004), which might also lead to a higher MLSP in cold adapted marine ectotherms by preventing oxidative damage of cellular structures.

The question arises whether the investigation of parameters like mitochondrial ageing, ROS generation, antioxidative defence and oxidative damage in the scallop species can explain why the polar species reaches a higher MLSP than the temperate species, because the difference in SMR between the two species is found only for young animals? Moreover, do the levels of ROS generation, antioxidative defence and oxidative damage contribute to the life prolonging effect of the lower SMR in the polar mud clam?

4.4. Mitochondrial ageing in marine bivalves

In the two temperate bivalve species *M. arenaria* and *A. opercularis* and in the Antarctic mud clam *L. elliptica* mitochondrial respiratory capacities and RCRs of isolated mitochondria decreased, whereas proton leak increased with age. For the first time this shows that mitochondrial ageing also takes place in marine bivalves. This is not an unexpected result if compared to studies of mitochondrial ageing in humans (Trounce et al., 1989; Cooper et al., 1992; Boffoli et al., 1994), other terrestrial vertebrates (Goodell and Cortopassi, 1998; Kokoszka et al., 2001; Hagen et al., 2002; Lopez-Torres et al., 2002; Ventura et al., 2002; Grattagliano et al., 2004) and invertebrates (Sohal et al., 1995a), which show similar trends. However, age related changes of mitochondrial functions have always been controversial. For example, Rasmussen et al. (2003) did not find any age related change in mitochondrial function in humans which is in contrast to many other studies of isolated mitochondria, and was explained by the preparation technique used in the study which was thought to prevent preparation damage to the mitochondria. However, Hagen et al. (1997) measured mitochondrial function in isolated rat cells and showed that the decline in mitochondrial function with age also took place when investigated in intact cells. They argued convincingly that mitochondrial deterioration with age cannot be attributed to damage during isolation.

In the present study, the directions of change in mitochondrial functions with age were similar in the investigated bivalve species. However, the extent and timescale of these changes were strikingly different between species.

4.4.1. Differences in mitochondrial ageing between M. arenaria and L. elliptica

Within the mud clam group (Publ. II), mitochondrial functions of the temperate shorter-lived *M. arenaria* changed more rapidly with age than those of the Antarctic longer-lived *L. elliptica*. This also holds true for the mitochondrial respiration and RCR when plotted against relative age, indicating a faster mitochondrial ageing in the temperate compared to the polar mud clam. The relative age of individual mud clams was calculated by using the maximum age found for both investigated populations and assuming this to be the maximum age that the population can reach. This assumption is supported by maximum lifespans found for *M. arenaria* and *L. elliptica* in the literature (Ralph and Maxwell, 1977; Winther and Gray, 1985; Urban and Mercuri, 1998; Strasser, 1999) and based on over 650 individual age determinations of *L. elliptica* sampled around King George Island (this study, M. Voigt (2004), Urban and Mercuri (1998) and Thomas Brey unpublished data).

Due to the close vicinity of the mitochondrial inner membrane and mt-DNA to the sites of oxygen radical generation, mitochondrial components are supposed to be prime targets of

ROS-dependent oxidation, which may lead to the decline in mitochondrial function with age (Shigenaga et al., 1994; Lenaz, 1998; Kowaltowski and Vercesi, 1999). We therefore hypothesized that an explanation for accelerated ageing of *M. arenaria* mitochondria may lie in higher rates of mitochondrial ROS formation in the temperate compared to the polar mud clam. Indeed, when investigating H_2O_2 generation rates of both species we found over 5-fold higher H_2O_2 generation rates (nmol H_2O_2 mg^{-1} mitochondrial protein min^{-1}) in *M. arenaria* mitochondria than in mitochondria isolated from *L. elliptica*. Hence, the more pronounced decrease in mitochondrial function with age in the temperate mud clam might be caused by ongoing oxidative damage. Our findings are in line with studies of insect and mammalian species, where higher ROS generation rates have been found in shorter- than longer-lived species (Ku et al., 1993; Sohal et al., 1995b). The activity of the mitochondrial enzymes cytochrome c oxidase (COX) and citrate synthase (CS) remained constant throughout all ages in *M. arenaria* and *L. elliptica*, indicating that in the investigated mud clams mitochondrial components other than these enzymes are hampered in their function by age-related oxidative damage.

With respect to the lower SMRs of the Antarctic *L. elliptica* compared to the temperate *M. arenaria* our results of lower H_2O_2 generation rates in *L. elliptica* would be coherent with the “Free Radical-Rate of Living” theory and the prediction of a lower ROS generation rate at lower SMR. At the same time, mitochondria isolated from *L. elliptica* showed generally higher aerobic capacities than mitochondria of *M. arenaria* individuals after the first 4 years of life. As H_2O_2 generation rates and oxygen consumption of the same mitochondrial suspension were measured in parallel, it was interesting to see that despite a lower mitochondrial oxygen consumption rate at older age and habitat temperature, H_2O_2 generation rates were still 5-fold higher in mitochondria isolated from *M. arenaria* than in mitochondria of *L. elliptica* individuals. *M. arenaria* therefore not only showed higher absolute amounts of H_2O_2 generation per mg protein than *L. elliptica,* but a higher fraction of consumed oxygen was converted to H_2O_2 ($\%H_2O_2/O_2$). With increasing age, H_2O_2 generation rates on the basis of mitochondrial protein remained stable in *M. arenaria*, whereas the percentage of oxygen converted to H_2O_2 steadily increased with age. As described by Lenaz (1998), reactive oxygen species generated by mitochondria may lead to the damage of mitochondrial structures like proteins, lipids and mtDNA which, in a vicious cycle, may enhance mitochondrial ROS generation and lead to even more oxidative damage. In *L. elliptica* absolute H_2O_2 generation rates as well as $\%H_2O_2/O_2$ declined significantly with age, which might show a better preservation of mitochondrial fitness in old *L. elliptica*. Comparative studies of rats and pigeons also reported lower $\%H_2O_2/O_2$ in isolated mitochondria of the long-lived pigeons compared to the shorter-lived rats, thereby corroborating our conclusions (Barja et al., 1994b). In aging *L. elliptica*

mitochondria our results show a protective reduction of ROS generation in relation to oxygen consumption.

A basic difference between *M. arenaria* and *L. elliptica* mitochondria is the higher proton leak in isolated mitochondria of the polar *L. elliptica* which according to the " uncoupling to survive" hypothesis from Brand (2000) could account for the lower rate of ROS generation in *L. elliptica* mitochondria. Porter et al. (1996) observed an increase in proton leak as a function of inner mitochondrial membrane surface area (cristae density) and found it also to be modulated by the degree of unsaturated fatty acids in the membrane. An increase in cristae density and/or elevated content of unsaturated membrane fatty acids has been observed in several marine cold adapted and cold acclimated ectotherms to cope with the effect of low temperatures on physical and biochemical processes like lower oxygen diffusion and membrane fluidity (Cossins et al., 1978; White and Somero, 1982; St.-Pierre et al., 1998; Guderley and St-Pierre, 2002). In the present study, the far higher activities of the mitochondrial- inner membrane bound enzyme cytochrome c oxidase (COX) in *L. elliptica* compared to *M. arenaria* may speak for a higher cristae density in the polar clam which would increase proton leak. The finding of similar levels of total unsaturated fatty acids in the soft tissue of *L. elliptica* and in several marine bivalves from warmer waters by Ahn et al. (2000) raises some doubt as to the contribution of membrane unsaturation in the higher proton leak in the polar clam. Altogether the polar *L. elliptica* might employ two defence strategies to suppress the production of hazardous ROS and gain higher MLSP: a low SMR at *in situ* temperatures ("Free Radical-Rate of Living" theory) and mild uncoupling by an elevated mitochondrial proton leak ("Uncoupling to survive" theory), which may be due to mitochondrial adaptations to the Antarctic temperature regime.

As set forth in chapter 4.5, *L. elliptica* additionally showed higher antioxidant capacities compared to *M. arenaria,* which may neutralize ROS release from the mitochondria, and presumably contributes to the higher MLSP of the polar species.

4.4.2. Mitochondrial ageing in swimming and burrowing bivalves: a case study comparing a temperate scallop to a temperate mud clam

The temperate scallop *A. opercularis* has a MLSP of 8-10 years, the shortest MLSP of all four investigated bivalve species. Compared to the results obtained in the two mud clams, one might expect a more pronounced decline in mitochondrial functions with age and the highest ROS generation rates of all species to occur in *A. opercularis*. In contrast, mitochondrial respiration, RCR and proton leak showed only marginal changes with age (Publ. III). Moreover, H_2O_2 generation rates with either succinate plus rotenone or pyruvate and malate (without rotenone) were next to not measurable in *A. opercularis*

despite high mitochondrial respiration rates at a lower proton leak (33%) than in *M. arenaria* (Publ. II). High mitochondrial capacity and lower proton leakage in the swimming *A. opercularis* compared to the burrowing *M. arenaria* reflects the more active lifestyle of the scallop. However a lower leak will, according to Brand (2000), account for higher rather than lower ROS generation.

This raises the question whether special mechanisms prevent the generation or the release of mitochondrial ROS in the temperate scallop. Indeed this could be true for scallops in general, as a small number of measurements in isolated mitochondria from *A. colbecki* at 0°C resulted in H_2O_2 generation rates below detection limits. Speaking for lower generation instead of higher scavenging capacity of mitochondrial ROS, are the lower tissue redox state (GSSG:GSH) and lower or comparable antioxidant enzyme activities in the scallops compared to the mud clams (Publ. I and III, Chapter 4.5). This indicates that the more active scallop lifestyle does not imply vastly higher mitochondrial ROS formation. Still, with the data obtained in the present study the biochemical mechanisms of high oxygen consumption and low ROS generation in scallop mitochondria remain unexplained.

The minor decrease in mitochondrial capacity with increasing age in *A. opercularis* stays in contrast to the dramatic decrease in *M. arenaria* mitochondria (Fig. 4.5) and may speak for a slower mitochondrial senescence in the scallop, despite the lower MLSP of *A. opercularis* compared to *M. arenaria*. However, COX and CS activities decreased with age in *A. opercularis* while activities remained constant in *M. arenaria*. These findings could indicate a decrease in mitochondria density over time in the mantle tissue of *A. opercularis*. The scallop may selectively remove damaged mitochondria from the cells and keep only highly functional mitochondria. This however remains speculative and has to be investigated in further studies. Age-related changes in mitochondrial numbers have up to now only been reported for human liver and a decrease in mitochondrial numbers per cell with age was observed (Tauchi and Sato, 1968). An active lifestyle as seen in actively swimming scallops might require the maintenance of cellular functions on a high level as long as possible to ensure a maximum scope for activity throughout life.

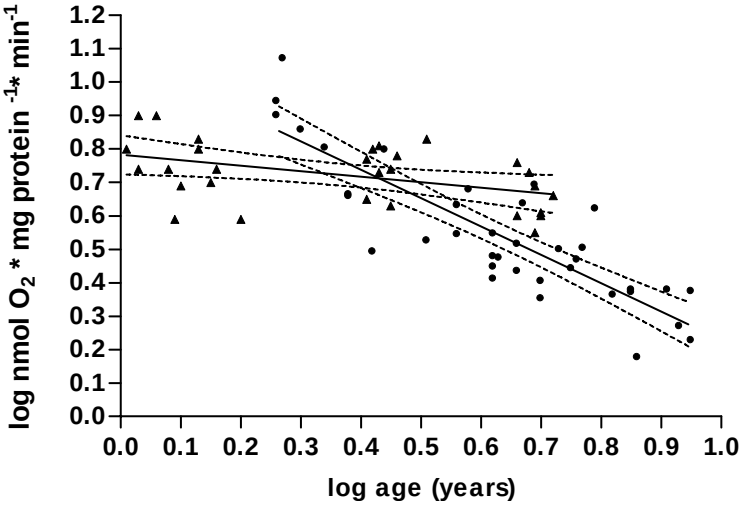

Fig. 4.5: State 3 respiration of mitochondria (R_M) isolated from *M. arenaria* (filled circle, N = 37, age range = 2-9 years) and *A. opercularis* (filled triangles, N = 29, age range = 1-5 years) mantle tissue vs. chronological age. Measurements were carried out at mean *in situ* temperature 10°C. Data represent 1 or the mean of 2-3 replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *M. arenaria*: $\log(R_M) = -0.847 * \log(age) + 1.076$, $r^2 = 0.730$, $p < 0.001$; *A. opercularis*: $\log(R_M) = -0.1639 * \log(age) + 0.783$, $r^2 = 0.168$, $p = 0.0168$.

In the scallop *Chlamys islandica,* reduced CS activity and mitochondrial capacity caused a decrease of adductor muscle aerobic capacity in mature compared to immature animals, which resulted in delayed recovery after exhaustive swimming (Brokordt et al., 2000). *A. opercularis* is a very active swimmer to avoid predation by starfish, crustaceans and man (Veale et al., 2000a; Veale et al., 2000b; Jenkins et al., 2003). Even small decreases in mitochondrial capacity and in COX and CS activities (mitochondrial number?) with age in *A. opercularis* may reduce tissue oxidative capacity and therefore swimming activity or recovery capacity, thereby threatening the animals' capacity to escape from danger (predators, trawls). This may cause an increase of extrinsic mortality (= elevated susceptibility to predation) in aged animals. In line with these considerations Brand (1991) recorded a gradual loss of activity in larger (older) *A. opercularis* which swim less often and require a stronger stimulus to elicit the swimming response.

Short MLSP despite slower mitochondrial senescence might be characteristic for active epifaunal species, highly prone to predation. These conclusions are in line with theoretical considerations by Williams and Day (2003) who postulate a selective effect of interactive extrinsic mortality causes, like predation, resulting in a slower physiological senescence of the species. Recently Reznick et al. (2004) developed a similar picture of reduced senescence, based on mortality and reproduction data, in goby fish from a high-predation

compared to a low-predation site, where the probability of surviving for 6 months is 20-30 times greater than in the high predation site.

4.4.3. Differences in mitochondrial ageing between A. opercularis and A. colbecki

As living *A. colbecki* were not available to investigate changes in mitochondrial functions with age, differences in mitochondrial ageing patterns between the two scallop species remain unknown. However, the activities of the mitochondrial enzymes COX and CS, measured in mantle tissue of individuals with different ages from both species, might nevertheless indicate a difference in mitochondrial ageing of the two scallop species (Publ. III). While COX and CS activities declined significantly with increasing age in the temperate *A. opercularis*, values remained stable in the Antarctic *A. colbecki*. This indicates that *A. colbecki* may better preserve tissue aerobic capacity over time so that the onset of weakening with respect to muscular performance may be postponed. This may also shift the onset of increased extrinsic mortality to older ages and support the higher MLSP of the Antarctic compared to the temperate scallop. The delayed onset of extrinsic mortality in *A. colbecki* might not only be crucial for old individuals to reach a high MLSP, but also for young individuals, as they live attached to the adults until they reach maturity and are therefore dependent on the survival of older individuals.

4.5. Age dependent antioxidative capacity and oxidative damage

4.5.1. Antioxidative capacity

Antioxidant enzymes like superoxide dismutase and catalase and small molecular antioxidants like glutathione and ascorbate remove reactive oxygen species from the cell to protect cell contents from oxidation. On first sight one would expect a positive correlation between antioxidant capacity and maximum lifespan. However, several studies of terrestrial vertebrates found no clear relationship between antioxidant capacity and MLSP. Barja et al. (1994a) examined antioxidative capacities in 7 different ectothermal and endothermal vertebrate species and found a negative correlation between superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and ascorbic acid and MLSP. Sohal et al (1990) on the other hand investigated 6 mammalian species and found a positive correlation of SOD and catalase activity with MLSP, whereas glutathione concentration was negatively correlated with MLSP and glutathione peroxidase activity did not show a conclusive pattern. Perez-Campo et al. (1998) compiled data from mammalian species and found a negative correlation of antioxidant capacity with MLSP.

Hence these studies give no conclusive answer about the relationship of antioxidative capacity and MLSP but demonstrate the straight forward idea of a positive correlation

between MLSP and antioxidative capacities to be incorrect. According to the “Free Radical-Rate of Living” theory, MLSP is negatively correlated with standard metabolic rate and ROS generation, respectively. Long living species might therefore not need high antioxidative capacities, as there are no ROS to fight against.

Antioxidant capacities of marine ectothermal vertebrates and invertebrates were extensively measured in various studies and wide-ranging species specific but also tissue specific differences were found (Viarengo et al., 1995; Buchner et al., 1996; Zielinski and Pörtner, 2000; Estevez et al., 2002; Correia et al., 2003). Up to now a correlation of antioxidative defence and MLSP has not been applied to marine ectotherms. Zielinski and Pörtner (2000) compared SOD and catalase activity of 11 marine ectothermal vertebrates and invertebrates with the corresponding metabolic rates and did not find a conclusive pattern.

The comparison of antioxidant capacities in marine ectothermal vertebrates and invertebrates is hampered by several factors. One major factor is the choice of tissue. It has been shown that different tissues, like muscle and liver in fish, and gills and digestive gland in bivalves, exhibit up to 10-fold differences in enzyme activities or in the concentrations of small molecular antioxidants (Viarengo et al., 1995; Abele et al., 1998; Pannunzio and Storey, 1998; Zielinski and Pörtner, 2000; Pena-Llopis et al., 2003). When comparing antioxidant capacities of different species, it is therefore crucial to investigate similar tissues, which in case of marine ectotherms is quite complicated, for example when including invertebrates like worms in comparison with bivalves and fish. Nevertheless, within certain boundaries, a between species comparison should be allowed within individual classes of molluscs or fish. In all cases the differences in lifestyles (e.g. mobility, food uptake) should be taken into account, which may be just as crucial for such comparisons as a close phylogenetic relationship.

The present data set allows comparison of the activities of the antioxidant enzymes superoxide dismutase and catalase and of the content of the small molecular antioxidant glutathione in mantle tissue of the four bivalve species (Publ. I and III).

Within the mud clam and scallop groups, the longer-lived species of each group (*L. elliptica, A. colbecki*) showed higher glutathione concentrations compared to their shorter-lived counterparts (*M. arenaria, A. opercularis*) (Fig. 4.6).

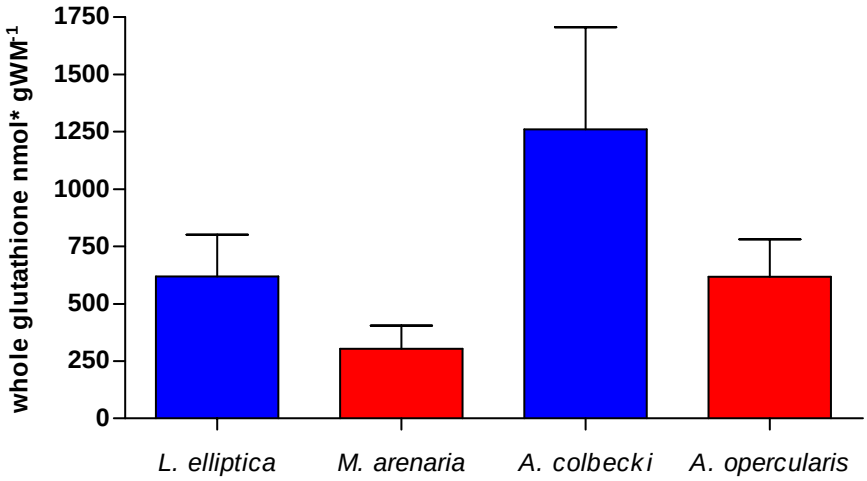

Fig. 4.6: Whole Glutathione content (2 * GSSG+GSH) in mantle tissue of the bivalves: *L. elliptica* (N = 29, age range = 2-22 years), *M. arenaria* (N = 23, age range = 2-8 years), *A. colbecki* (N = 26, age range = 3-14 years), *A. opercularis* (N = 28, age range =1-5 years).

A similar picture has been found in liver samples of two zoarcid fish (Heise et al., in rev). The longer-lived polar *Pachycara brachycephalum* showed higher glutathione concentrations compared to the temperate shorter-lived *Zoarces viviparus* (Heise et al., in rev and E. Brothe pers. comm. for differences in MLSP of fish species). Such a clear picture could not be found for the activities of antioxidant enzymes (Fig. 4.7). SOD activities (Fig. 4.7A) within the two groups (mud clams, scallops) were slightly lower in the longer-living compared to the shorter-lived species of each group while no general pattern was found concerning catalase activities (Fig. 4.7B). The Antarctic *L. elliptica* showed vastly higher catalase activities compared to the temperate *M. arenaria*, whereas in the scallop group the temperate *A. opercularis* showed slightly higher activities compared to the Antarctic *A. colbecki*. No significant difference could be found between the two zoarcid fishes, *P. brachycephalum* and *Z. viviparus*, when comparing SOD activities of liver samples (Heise et al., in rev). Zielinski and Pörtner (2000) found lower catalase and SOD activities in a shorter-lived compared to a longer-lived cephalopod species.

Like in the studies of terrestrial vertebrates no conclusive relationship in general could therefore be found between MLSP and the levels of antioxidant enzyme activities. In contrast, glutathione seems to be a good indicator which distinguishes short- from long-lived species of the same lifestyle. In the present study the actively swimming scallops displayed higher glutathione contents than the burrowing mud clams. Lifestyle might thus be a factor that has to be taken into account when comparing the glutathione concentration of different species.

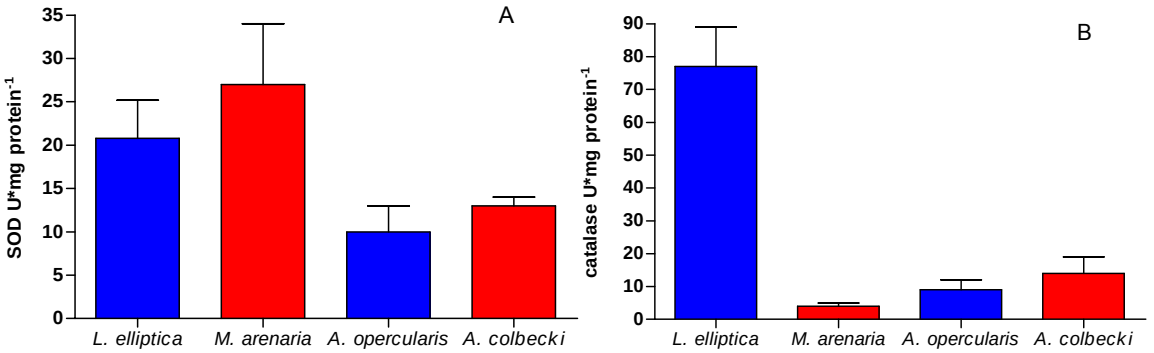

Fig. 4.7: Activities of the antioxidant enzymes superoxide dismutase (A) and catalase (B) in mantle tissue of all four bivalves measured over all ages at 20°C refererence temperature: *L. elliptica* (N= 29 catalase, 15 SOD, age range: 2-28 years catalase, 5-28 years SOD), *M. arenaria* (N=16 catalase, 17 SOD, age range: 2-8y), *A. colbecki* (N=29 catalase, N=5 SOD, age range: 2-16 years catalase, 4-8 years SOD) *A. opercularis* (N= 25 catalase, N= 27 SOD, age range: 1-5 years).
Q_{10} catalase: *L. elliptica*: 1.8; *M. arenaria*: not detected; *A. opercularis*: 1.57; *A. colbecki*: 1.37; Q_{10} superoxide dismutase: all species around 1.

A review of oxidative stress in polar and temperate marine ectotherms by Abele and Puntarulo (2004), showed a general trend towards higher antioxidant capacities, especially higher levels of non-enzymatic compounds, in polar compared to temperate marine ectotherms. As all longer-lived species of the present study were taken from Antarctica, future studies should compare long- and short-lived temperate bivalves and analyse whether the positive relationship between MLSP and glutathione content still holds true. A good candidate for a long-lived temperate bivalve could be *Arctica islandica*, a common member of the North and Baltic Sea, where individuals with an age up to 155 years have been found (Witbaard, 1997). This species could be compared with the shorter-lived *M. arenaria* (MLSP ~13 years) from the North or Baltic Sea, as they belong to the same ecotype (filter feeding, burrowing).

An additional factor complicating an interspecies comparison of antioxidative capacities is the change of antioxidant enzyme activities and of the concentration of low molecular antioxidants e.g. glutathione and ascorbate with age as seen in studies of marine ectotherms (Appendix) and terrestrial vertebrates and invertebrates (Sestini et al., 1991; Leeuwenburgh et al., 1994).

These findings suggest that comparisons of antioxidant capacities or other parameters in different species should be carried out in individuals which exhibit the same relative age, i.e. reached the same percentage of their maximum lifespan. For example, Viarengo et al.

(1995) found similar levels of GSH in the longer-lived *A. colbecki* (MLSP~45 years) as in the shorter-lived *Pecten jacobäus* (MLSP~10 years, Margus, 1991). In the light of the present results obtained in the bivalve species and of recent results obtained in the two zoarcid fish species, we would have expected a higher GSH content in *A. colbecki* compared to *P. jacobäus*. The study by Viarengo et al., however, compared individuals of both species with a shell height of 60 mm. According to the growth curve of Heilmayer et al. (2003), an individual of *A. colbecki* with 60 mm shell height is approximately 8 years old (~ rel age 17%) while according to Margus (1991) a mean shell height of 65 mm in *P. jacobaeus* typifies 3 years old individuals (~ rel age 30%). The present study showed a decrease in GSH concentration in *A. colbecki* with age, hence similar GSH concentrations in *A. colbecki* and *P. jacobäus* as found by Viarengo et al. (1995) could result from the comparison of individuals with different age, according to chronological or relative age, and might not show the true values for similarly aged individuals from both species.

In the present study, the analysis of antioxidative defence parameters and other parameters throughout the lifetime of different species, therefore, not only provides information about age-dependent changes but also allows an estimate of the true species-specific differences between these parameters.

The concentration of total glutathione (tGSH) in mantle tissue of *L. elliptica* and the two scallop species declined with age whereas in *M. arenaria* tGSH increased with age. The investigation of the ratio of GSSG:GSH, which is a marker of the oxidative status in the tissue, revealed higher and increasing values with age in *M. arenaria* whereas values were generally lower and declined or remained stable with age in the other 3 bivalve species.

High levels of tissue oxidative status and the increase in tissue oxidation with age despite the increase in tGSH in *M. arenaria* might reflect the higher H_2O_2 generation rate and the age-dependent increase in $\%H_2O_2/O_2$ of *M. arenaria* mitochondria. The lower tissue oxidative status and the stable or decreasing GSSG:GSH ratios with age in *L. elliptica* and the two clams, respectively, are in line with the low H_2O_2 generation rates of isolated mitochondria in the three species. Moreover, the decrease in GSSG:GSH ratio in *L. elliptica* might reflect the decrease in H_2O_2 generation rate with age. While catalase activities remained stable with age in the two mud clams, activities declined in the two scallop species, with a much more pronounced decrease in the short-lived *A. opercularis* compared to the longer-lived *A. colbecki*, showing a more rapid loss of antioxidant capacity with age in the temperate scallop compared to the other three species. Activities of the antioxidant enzyme SOD remained on a stable level with age in the two mud clams

and the two scallop species, implicating a continued need for stable superoxide anion scavenging throughout all ages in all four species.

Altogether, this interspecies comparison of bivalves indicates that the preservation of antioxidant capacity and a low oxidative status in the tissue might be crucial to control the physiological ageing process which co-determines MLSP.

4.5.2. Oxidative damage and age-related protein contents in mantle tissue

Despite the existence of antioxidative defence systems, a certain level of oxidative damage is inevitable. As a marker for lipid and protein oxidation, the concentration of the fluorescent age pigment lipofuscin and the concentration of protein carbonyls were investigated in mantle tissue of differently aged individuals of all four bivalve species. Additionally, the content of protein was measured in the mantle tissue to investigate whether a change in tissue composition occurred with increasing age in the different bivalves (Publ. I and III).

In *L. elliptica*, *A. colbecki* and *A. opercularis* lipofuscin accumulated with increasing age. In mantle tissue of *M. arenaria* the lipofuscin content increased only during the second half of the lifespan of this species, in individuals > 5 years of age. During the first half of their life lipofuscin concentrations even decreased with age.

A review of the existing literature on lipofuscin accumulation in marine ectotherms shows a general increase with age in invertebrates and vertebrates (Appendix). In contrast, Hole et al. (1995) observed higher lipofuscin concentrations in young (2-4 years) vs. old (> 10 years) *Mytilus edulis*, which was explained by a higher SMR of young compared to old animals, as an influence of the SMR on lipofuscin accumulation had been previously recorded by Sohal (1981) for *Drosophila melanogaster*. However, a decrease from relatively high initial lipofuscin concentrations during early lifetime can also be interpreted as a process of “rejuvenation” (Terman, 2001), associated with the dilution of waste material during intensive growth of the young animal. This may explain the decrease in lipofuscin accumulation in *M. arenaria* individuals < 4 years of age. In 5 years old specimens and older, a lower rate of dilution of damage due to age-related slower growth and cell division may enhance lipofuscin particle density.

Within the lifetime window of *M. arenaria* (Publ. I), higher lipofuscin concentrations were found in *M. arenaria* than in *L. elliptica*. Next to the influence of SMR on lipofuscin accumulation, the more oxidized redox state (GSSG:GSH) and the lower glutathione concentrations and catalase activities in *M. arenaria* compared to *L. elliptica* could be an explanation for the higher lipofuscin values in *M. arenaria*. In line with this consideration,

the polar and temperate scallop species showed lower lipofuscin concentrations together with lower GSH:GSSG ratios and higher glutathione concentrations, despite higher SMRs when compared to the polar and temperate mud clam species.

Within the scallop group, the temperate *A. opercularis* showed a more pronounced increase in lipofuscin levels than the Antarctic *A. colbecki*. This could reflect maintenance of cellular integrity and avoidance of waste accumulation in the longer-lived Antarctic scallop linked to the higher glutathione concentration and better preservation of antioxidant capacity, as seen in the less pronounced decrease in catalase activity with age. These results indicate that a high and preserved antioxidative capacity together with a generally low tissue redox state might be crucial to avoid extended oxidative damage of lipids and proteins and, in consequence, lipofuscin accumulation.

Protein carbonyl levels in the mud clam group showed a similar picture as found for lipofuscin. A continuous increase in protein carbonyl content with age was found In *L. elliptica*, whereas in *M. arenaria* concentrations decreased during the first 5 years and then seemed to remain at a constant value (see Discussion of Publ. I). Again the fast growth in young individuals of *M. arenaria* might lead to a dilution of protein carbonyl levels which slows down with age and may explain the relatively constant carbonyl concentrations in *M. arenaria* individuals older than 5 years. In *L. elliptica* the continuous increase in carbonyl concentration with age might, on the one hand, be the result of a species-specific strategy to preserve storage proteins despite protein oxidation (Discussion Publ. I, Ahn et al., 2003). On the other hand, the age-related increase in carbonyl levels could also be the cause of the increase in lipofuscin concentration, which has been found to impair proteasome related proteolysis of oxidised proteins (Sitte et al., 2000).

In parallel with the continuous increase in lipofuscin in *L. elliptica* and *A. opercularis* the protein content in the mantle tissue decreased with age, whereas in *M. arenaria* and *A. colbecki* stable or increasing protein concentrations could be observed together with a less pronounced or delayed increase in lipofuscin concentration. This may indicate a more pronounced age-related imbalance in protein synthesis and degradation in *L. elliptica* and *A. opercularis* compared to the other two bivalve species.

Age-related loss of muscle proteins is well documented in humans and rats (Holloszy et al., 1991; Booth et al., 1994), and a declining protein synthesis rate with age has been shown for mitochondrial and mixed muscle protein in vertebrates (human, mouse) and invertebrates (*Drosophila*) (Marcus et al., 1982; Bailey and Webster, 1984; Welle et al., 1993; Rooyackers et al., 1996; Yarasheski, 2003). Rooyackers et al. (1996) hypothesised that the decrease in the rates of mitochondrial protein synthesis is likely to be responsible

for the decline in the muscle oxidative capacity and mitochondrial function with age that he observed in human skeletal muscle. The decrease in protein content together with the decline in mitochondrial function and tissue oxidative capacity (decline in COX, CS activities) with age in *A. opercularis* supports this hypothesis. The results of *M. arenaria* and *L. elliptica* were less clear. *M. arenaria* showed stable protein concentration but a dramatic decrease in mitochondrial functions with age, whereas in *L. elliptica* a decrease in protein content was observed but mitochondrial functions declined less pronounced.

How and if the increase in lipofuscin and protein carbonyl levels and the decrease in protein contents with age impair physiological functions in the investigated bivalve species remains unclear, especially in the light of the high MLSP of *L. elliptica*. This has to be investigated in studies about the change in stress resistance and physical activity of differentially aged individuals of the different species.

5. CONCLUSIONS AND PERSPECTIVES

The present study addressed chronological and physiological ageing patterns and their mechanistic background in four bivalve species, two mud clam and two scallop species from the Antarctic and temperate environment, with the overall goal to identify mechanisms and strategies that lead to a longer lifespan in the polar compared to the temperate bivalve species.

Lower standard metabolic rates (SMR) in the longer-lived polar compared to the temperate mud clam are in line with the “Rate of Living” theory and may contribute to the higher MLSP in the polar species. However, a universal link between standard metabolic rate and maximum lifespan (MLSP) in bivalves cannot be postulated, as the scallop species did not display distinctly different SMRs throughout all ages. Low SMR is thus only one factor that can lead to extended life spans of marine ectotherms.

In both groups of bivalves, mud clams and scallops, the results of parameters characterising mitochondrial functions, antioxidative defence and oxidative damage indicate that the “Free Radical Theory of Ageing” can be applied to explain the physiological ageing process in bivalves:

I) The preservation of mitochondrial functions with age seems to be crucially important for a high MLSP:

The longer-lived polar mud clam showed a better preservation of mitochondrial functions with age compared to the shorter-lived temperate mud clam (Publ. II). Within the scallop group mitochondrial enzyme (CS, COX) activities remained stable over age in the longer-lived polar scallop, whereas in the shorter-lived temperate scallop the same activities significantly decreased with age (Publ. III).

II) The concentration and preservation of antioxidant capacity is obviously another factor that influences MLSP:

In both groups the longer-lived polar species had a higher concentration of the redox regulator and antioxidant glutathione compared to the temperate species. Moreover, in the mud clam group, tissue redox state was preserved on a more reduced level with age in the longer-lived compared to the shorter-lived species. Furthermore, the two polar species displayed far higher (*L. elliptica*) or better conserved (*A. colbecki*) activities of the H_2O_2-detoxifying antioxidant enzyme catalase with age.

III) The avoidance of waste accumulation (lipofuscin, protein carbonyls) and the conservation of tissue composition (protein content) are not absolutely crucial for reaching higher MLSP:

In the scallop group avoidance of waste accumulation (lipofuscin) and conservation of tissue protein content are linked to higher MLSP, whereas in the mud clam group these factors do not appear to affect MLSP determination.

Importantly this study documents that the investigated Antarctic and temperate bivalve species do not succumb to a uniform ageing process, which simply proceeds more slowly at cold Antarctic temperatures and thereby produces a higher MLSP in the cold. Likewise, the shorter MLSP of the actively swimming temperate scallop compared to the less active temperate mud clam is not simply the result of the same ageing programme advancing at faster pace because higher activity levels produce a stoichiometric increase of mitochondrial radical production.

The results lead to the conclusion that the absolute values of the investigated physiological parameters and the changes of these parameters with age reflect the species specific adjustments to (**IV**) the environmental temperature regime, (**V**) lifestyle (mobility) and thus (**VI**) the ecological niche of each species:

(**IV**) Maintenance of mitochondrial function and low levels of tissue oxidation status and of ROS production are caused by mild uncoupling in the polar mud clam (*L. elliptica*), and this strategy might result from the animal's biochemical adaptation to Antarctic water temperatures (high cristae densities?).

(**V**) In the temperate scallop a marginal decrease in mitochondrial function with age and the low level of tissue oxidation status (GSSG:GSH) also relates to low rates of mitochondrial ROS generation. In this case low ROS generation may result from adaptation to the active lifestyle, which requires highly functional, well coupled mitochondria, characterized by a high RCR and low proton leak and at the same time by a very low production rate of detrimental ROS. The exact mechanisms underlying the extremely low rates of ROS generation remain unclear.

(**VI**) Slow mitochondrial senescence in the temperate scallop compared to the temperate mud clam further indicates that interactive extrinsic mortality might also be a factor influencing the MLSP of the species and support different ageing strategies.

While the temperate mud clam seems to tolerate some mitochondrial senescence and to follow a strategy of "tear and wear", getting old without high costs for reparation and renewal of cell components, the scallop cannot allow senescence of mitochondria due to its active lifestyle. So it pursues the "superman" strategy, which means to keep as fit as possible and to evade extrinsic mortality from predation as long as possible. It would be interesting to monitor the physiological ageing process of "old" *A. opercularis* individuals

taken from the environment, to see whether the animals would still die at an age of 8-10 years, or if they would reach older ages than predicted in the literature when provided with a safe, predator-free environment (Fig. 5.1). Reznick et al. (2004) for example found that guppies from a high predation site lived up to 35% longer than guppies from a low predation site, when both groups were reared in an aquarium.

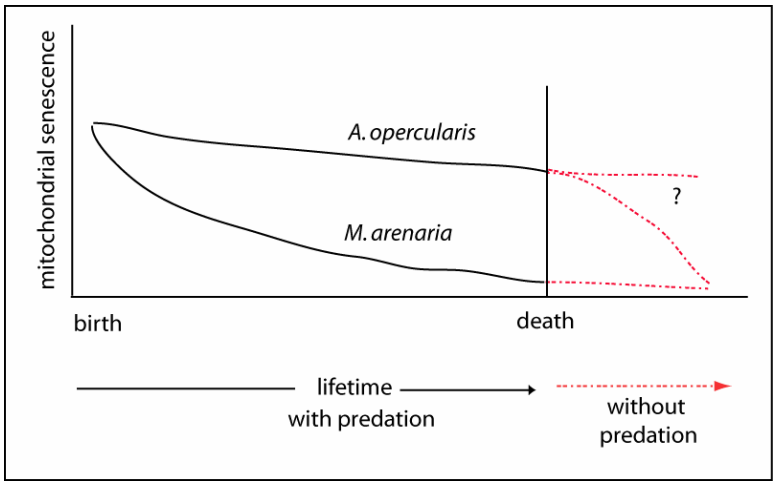

Fig. 5.1: Scheme of mitochondrial ageing in the temperate mud clam *M. arenaria* and scallop *A. colbecki*.

For further research in the field of bivalve ageing it should be taken into account that chronological age, i.e. lifetime in years, is a parameter which is easily determined by individual growth checks or from shell size using a population specific VBGF. The physiological age is more difficult to determine, as it is a less defined parameter, based on the analysis of physiological processes and biochemical patterns to describe the physiological fitness of an individual at a certain chronological age. In the present study the determination of physiological age was based on different physiological parameters, chosen with respect to the “Rate of Living” theory by Pearl (1928), “Free Radical Theory of Ageing” by Harman (1956) and the “Uncoupling to Survive” hypothesis by Brand (2000). As some parameters display different trends with age in the different species, only a complex survey of a whole set of parameters can give an idea of the change in the overall physiological state of an animal with age. The effect that changes of physiological parameters with age have on whole animal physiological fitness however, remains unknown.

As a future perspective for aging studies in bivalves and presumably other marine invertebrates, tests of physiological fitness should be carried out at the whole animal level

for example by investigating age dependent swimming and fleeing capacity or age dependent tolerance towards environmental stress factors like rapid temperature increments. It should be a future aim to investigate how age-related changes in cellular parameters affect whole animal physiological fitness and how theses changes in fitness determine MLSP of a population.

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Changes in Antioxidative capacity and oxidative damage parameters with age in marine vertebrates and invertebrates

Species group	Species	Organ	Temperature regime	Parameter measured	Effect	Ref
crustacea	*Gammarus locusta*	Whole body	Temperate	Catalase	No change	6
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	Catalase	Decrease	9
crustacea	*Aristeus antennatus* (males)	Brain	Cold	Catalase	Decrease	12
crustacea	*Aristeus antennatus* (females)	Brain	Cold	Catalase	Decrease	12
cephalopoda	*Sepia officinalis*	Mantle	Warm	Catalase	Decrease	13
cephalopoda	*Sepia officinalis*	Gills	Warm	Catalase	Decrease	13
bivalvia	*Laternula elliptica*	Mantle	Cold	Catalase	No change	This study
bivalvia	*Mya arenaria*	Mantle	Temperate	Catalase	No change	This study
bivalvia	*Aequipecten opercularis*	Mantle	Cold Temperate	Catalase	Decrease	This study
bivalvia	*Adamussium colbecki*	Mantle	Cold	Catalase	No change	This study
polychaeta	*Arenicola marina*	Chloragog tissue	Temperate	Catalase	Increase	14
bivalvia	*Mytilus edulis*	Whole body	Cold Temperate	Catalase	Decrease (size depend.)	3
crustacea	*Gammarus locusta*	Whole body	Temperate	SOD	Decrease	6
teleost fish	*Oncorhynchus mykiss*	Muscle tissue	Cold freshwater	SOD	No change	7
teleost fish	*Dicentrarchus labrax*	Muscle tissue	Temperate	SOD	No change	7
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	SOD	No change	9
crustacea	*Aristeus antennatus* (males)	Brain	Cold	SOD	Increase	12
crustacea	*Aristeus antennatus* (females)	Brain	Cold	SOD	Decrease	12
cephalopoda	*Sepia officinalis*	Mantle	Warm	SOD	Increase	13
cephalopoda	*Sepia officinalis*	Gills	Warm	SOD	Increase	13
bivalvia	*Laternula elliptica*	Mantle	Cold	SOD	No change	This study
bivalvia	*Mya arenaria*	Mantle	Temperate	SOD	No change	This study
bivalvia	*Aequipecten opercularis*	Mantle	Cold Temperate	SOD	No change	This study
polychaeta	*Arenicola marina* (winter)	Chloragog tissue	Temperate	SOD	Decrease	14
polychaeta	*Arenicola marina* (summer)	Chloragog tissue	Temperate	SOD	Increase	14
crustacea	*Gammarus locusta*	Whole body	Temperate	GPX	Decrease	6
teleost fish	*Oncorhynchus mykiss*	Muscle tissue	Cold freshwater	GPX	No change	7
teleost fish	*Dicentrarchus labrax*	Muscle tissue	Temperate	GPX	No change	7
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	GPX	Decrease	9

Species group	Species	Organ	Temperature regime	Parameter measured	Effect	Ref
crustacea	*Aristeus antennatus* (males)	Brain	Cold	GPX	Increase	12
crustacea	*Aristeus antennatus* (females)	Brain	Cold	GPX	No change	12
cephalopoda	*Sepia officinalis*	Mantle	Warm	GPX	Increase	13
cephalopoda	*Sepia officinalis*	Gills	Warm	GPX	Increase	13
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	Glutathione	Decrease	1
bivalvia	*Mytilus edulis*	Gillss	Temperate	Glutathione	Decrease	1
teleost fish	*Oncorhynchus mykiss*	Muscle tissue	Cold freshwater	Glutathione	No change	7
teleost fish	*Dicentrarchus labrax*	Muscle tissue	Temperate	Glutathione	No change	7
bivalvia	*Latermula elliptica*	Mantle	Cold	Glutathione	Decrease	This study
bivalvia	*Mya arenaria*	Mantle	Temperate	Glutathione	Increase	This study
bivalvia	*Aequipecten opercularis*	Mantle	Cold Temperate	Glutathione	Decrease	This study
bivalvia	*Adamussium colbecki*	Mantle	Cold	Glutathione	Decrease	This study
polychaeta	*Arenicola marina*	Chloragog tissue	Temperate	Glutathione	No change	14
teleost fish	*Oncorhynchus mykiss*	Muscle tissue	Cold freshwater	Vitamine C	Decrease	7
teleost fish	*Dicentrarchus labrax*	Muscle tissue	Temperate	Vitamine C	Decrease	7
teleost fish	*Oncorhynchus mykiss*	Muscle tissue	Cold freshwater	Vitamine E	Decrease	7
teleost fish	*Dicentrarchus labrax*	Muscle tissue	Temperate	Vitamine E	Decrease	7
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	Vitamine E	Decrease	8
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	Carotenoids	Decrease	9
bivalvia	*Mytilus edulis*	Whole body	Cold Temperate	Lipofuscin	Increase	3
bivalvia	*Mytilus edulis*	Digestive gland	Temperate	Lipofuscin	Decrease	10
cephalopoda	*Sepia officinalis*	Mantle	Warm	Lipofuscin	Increase	13
cephalopoda	*Sepia officinalis*	Gills	Warm	Lipofuscin	Increase	13
bivalvia	*Latermula elliptica*	Mantle	Cold	Lipofuscin	Increase	This study
bivalvia	*Mya arenaria*	Mantle	Temperate	Lipofuscin	Decrease to Increase	This study
bivalvia	*Aequipecten opercularis*	Mantle	Cold Temperate	Lipofuscin	Increase	This study
bivalvia	*Adamussium colbecki*	Mantle	Cold	Lipofuscin	Incraese	This study
crustacea	*Waldeckia obesa*	Brain	Cold	Lipofuscin	Increase	15
gastropoda	*Eurhomalea exalbida*	Connective tissue	Cold	Lipofuscin	Increase	16
gastropoda	*Monodonta lineata*	Cerebral ganglia and digestive gland	Temperate	Lipofuscin	No trend	17
crustacea	*Callinectes sapidus*	Eye stalk	Temperate	Lipofuscin	Increase	18

Species group	Species	Organ	Temperature regime	Parameter measured	Effect	Ref
crustacea	*Homarus gammarus*	Brain	Temperate	Lipofuscin	Increase	From 19
crustacea	*Panulirus cygnus*	Brain	Warm	Lipofuscin	Increase	From 19
crustacea	*Cherax quadricarinatus*	Brain	Warm	Lipofuscin	Increase	From 19
crustacea	*Cherax scuspidatus*	Brain	Temperate	Lipofuscin	Increase	From 19
crustacea	*Pacifastacus leniusculus*	Brain	Temperate	Lipofuscin	Increase	From 19
teleost fish	*Oncorhynchus mykiss*	Muscle tissue	Cold freshwater	Protein carbonyls	Increase	7
teleost fish	*Dicentrarchus labrax*	Muscle tissue	Temperate	Protein carbonyls	Increase	7
bivalvia	*Laternula elliptica*	Mantle	Cold	Protein carbonyls	Increase	This study
bivalvia	*Mya arenaria*	Mantle	Temperate	Protein carbonyls	Decrease	This study
bivalvia	*Aequipecten opercularis*	Mantle	Cold Temperate	Protein carbonyls	No change	This study
bivalvia	*Adamussium colbecki*	Mantle	Cold	Protein carbonyls	No change	This study

Environmental and lifestyle information of species in table 1

Species group	Species	MLSP	Measured age range	Location	Temperature range	Lifestyle	Ref
bivalvia	*Adamussium colbecki*	~45y	3-14years	Terra Nova Bay, Antarctica	-1.9 – 1	swimming	This study
bivalvia	*Aequipecten opercularis*	8-10y	1-5years	Irish Sea, Isle of Man	7.4 – 13.7	swimming	This study
bivalvia	*Latermula elliptica*	36y	2-28years	South Shetl. Isl., Antarctica	-1.9- 2°C	burrowing	This study
bivalvia	*Mytilus edulis*	18-24y	Groups: 2-4; 6-8; >10	Linher River Plymouth, UK	4-18°C (Ref 2)	sessile	9
bivalvia	*Mytilus edulis*	20y	2-10years	White sea, Russia	-0.9-13.8°C	sessile	3
bivalvia	*Mytilus edulis*	18-24y	Groups: 2-4; 6-8; >10	Beggars Island Plymouth, UK	4-18°C (Ref 2)	sessile	1 + 8 + 10
bivalvia	*Mya arenaria*	~13y	0-9y	North Sea, Netherlands	-1 to +20°C	burrowing	This study
cephalopoda	*Sepia officinalis*	14month in captivity	1.5-12 month	Texas, aquaculture	20-22°C	swimming	13
crustacea	*Aristeus antennatus*	10y (Ref 11)	Size groups	Mediterranian SE, Spain	Deep water 700m	crawling	12
crustacea	*Callinectes sapidus*	?	9month – 2years	Chesapeak Bay, USA	5-30°C and constant 20°C in laboratory	crawling	18
crustacea	*Cherax quadricarinatus*	3-5y	Up to 25month	Coral Sea, Australia	14-30°C	crawling	From 19
crustacea	*Cherax scuspidatus*	8 y	Up to 7 month	Coral Sea, Australia	Mean annual 16,9	crawling	From 19
crustacea	*Gammarus locusta*	22 w (Ref 5)	groups 1-4; 4-7; >7 weeks	Sado estuary, Portugal	11-24°C (Ref 4)	epibenthic	6
crustacea	*Homarus gammarus*	40-50y	4,4-9,6years	England, Wales, Scotland	Mean annual 10°C	crawling	From 19
crustacea	*Pacifastacus leniusculus*	12-16y	Up to 6.5 years	Lake Mälaren, Sweden	Mean annual 9.1	crawling	From 19
crustacea	*Panulirus cygnus*	27y	1-6years	West and South West, Australia	Mean annual 21.5	crawling	From 19
crustacea	*Waldeckia obesa*	?	0-6years	Weddell Sea, Antarctica	-2.2	Swimming?	15
teleost fish	*Dicentrarchus labrax*	6y	1, 3 and 5 years	Central Thyrrhenian Sea	Temperate	swimming	7
teleost fish	*Oncorhynchus mykiss*	5y	3month, 1,2,and 3years	Aquaculture	11.8	swimming	7
gastropoda	*Eurhomalea exalbida*	>70y	0-70years	Ushuaia Bay, Chile	4.5-8.7	crawling?	16
gastropoda	*Monodonta lineata*	>10.5y	1.5–10.5 yr	Widemouth Bay, UK	??	crawling	17
polychaeta	*Arenicola marina*	?	Young adult	Wadden Sea, Germany	-1- 20°C	burrowing	14

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Bremerhaven, den 07. 02. 2005

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Erklärung gem. § 5 (1) Nr. 3 PromO

Ich erkläre hiermit,

1. dass ich mich vor dem jetzigen Promotionsverfahren keinem anderen Promotionsverfahren unterzogen habe

und

2. dass ich außer dem jetzt laufenden Promotionsverfahren auch kein anderes beantragt habe.

Eva Philipp