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Life strategies in the long-lived bivalve *Arctica islandica* on a latitudinal climate gradient – Environmental constraints and evolutionary adaptations

Lebensstrategien der langlebigen Muschel *Arctica islandica,* untersucht an Populationen entlang eines Klimagradienten – Umwelteinflüsse und evolutionäre Anpassungen

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FREQUENTLY USED ABBREVIATIONS

ADP	Adenosine diphosphate
AFDM	Ash free dry mass
ATP	Adenosine triphosphate
B	Burrowed
BrdU	5-Bromo-2-deoxyuridine
CAT	Catalase
CS	Citrate synthase
EC	Energy charge
GB	German Bight
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GSx	Total glutathione (GSH + 2 x GSSG)
IC	Iceland
LDH	Lactate dehydrogenase
MLSP	Maximum life span potential
MRD	Metabolic rate depression
MSR	Mass specific respiration
NE	North East
NW	North West
ODH	Octopine dehydrogenase
PO_2	Oxygen partial pressure
P_{crit}	Critical PO_2
NO	Nitric oxide
NOS	Nitric oxide synthase
RLU	Relative luminescence units
ROS	Reactive oxygen species
SMR	Standard metabolic rate
SOD	Superoxide dismutase
SST	Sea surface temperature
VBGF	Von Bertalanffy growth function

SUMMARY

The ocean quahog, *Arctica islandica* is the longest-lived non-colonial animal known to science. A maximum individual age of this bivalve of 405 years has been found in a population off the north western coast of Iceland. Conspicuously shorter maximum lifespan potentials (MLSPs) were recorded from other populations of *A. islandica* in European waters (e.g. Kiel Bay: 30 years, German Bight: 150 years) which experience wider temperature and salinity fluctuations than the clams from Iceland.

The aim of my thesis was to identify possible life-prolonging physiological strategies in *A. islandica* and to examine the modulating effects of extrinsic factors (e.g. seawater temperature, food availability) and intrinsic factors (e.g. species-specific behavior) on these strategies. Burrowing behavior and metabolic rate depression (MRD), tissue-specific antioxidant and anaerobic capacities as well as cell-turnover (= apoptosis and proliferation) rates were investigated in *A. islandica* from Iceland and the German Bight. An inter-species comparison of the quahog with the epibenthic scallop *Aequipecten opercularis* (MLSP = 8-10 years) was carried out in order to determine whether bivalves with short lifespans and different lifestyles also feature a different pattern in cellular maintenance and repair.

The combined effects of a low-metabolic lifestyle, low oxidative damage accumulation, and constant investment into cellular protection and tissue maintenance, appear to slow-down the process of physiological aging in *A. islandica* and to afford the extraordinarily long MLSP in this species. Standard metabolic rates were lower in *A. islandica* when compared to the shorter-lived *A. opercularis.* Furthermore, *A. islandica* regulate mantle cavity water PO_2 to mean values < 5 kPa, a PO_2 at which the formation of reactive oxygen species (ROS) in isolated gill tissues of the clams was found to be 10 times lower than at normoxic conditions (21 kPa).

Burrowing and metabolic rate depression (MRD) in Icelandic specimens were more pronounced in winter, possibly supported by low seawater temperature and food availability, and seem to be key energy-saving and life-prolonging parameters in *A. islandica.* The signaling molecule nitric oxide (NO) may play an important role during the onset of MRD in the ocean quahog by directly inhibiting cytochome-*c*-oxidase at low internal oxygenation upon shell closure. In laboratory experiments, respiration of isolated *A. islandica* gills was completely inhibited by chemically produced NO at low experimental $PO_2 \leq 10$ kPa. During shell closure, mantle cavity water PO_2 decreased to 0 kPa for longer than 24 h, a state in which ROS production is supposed to subside. Compared to other mollusk species, onset of anaerobic metabolism is late in *A. islandica* in the metabolically reduced state. Increased accumulation of the anaerobic metabolite succinate was initially detected in the adductor muscle of the clams after 3.5 days under anoxic incubation or in burrowed specimens. A ROS-burst was absent in isolated gill tissue of the clams following hypoxia (5 kPa)-reoxygenation (21 kPa). Accordingly, neither the activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), nor the specific content of the ROS-scavenger glutathione (GSH) was enhanced in different tissues of the ocean quahog after 3.5 days of self-induced or forced hypoxia/anoxia to prepare for an oxidative burst.

While reduced ROS formation compared to routine levels lowers oxidative stress during MRD and also during surfacing, the general preservation of high cellular defense and the efficient removal and replacement of damaged cells over lifetime seem to be of crucial importance in decelerating the senescent decline in tissues of *A. islandica.* Along with stable antioxidant protection over 200 years of age, proliferation rates and apoptosis intensities in most investigated tissues of the ocean quahog were low, but constant over 140 years of age. Accordingly, age-dependent accumulations of protein and lipid oxidation products are lower in *A. islandica* tissues when compared to the shorter-lived bivalve *A. opercularis*.

The short-lived swimming scallop is a model bivalve species representing the opposite life and aging strategy to *A. islandica.* In this species permanently high energy throughput, reduced investment into antioxidant defense with age, and higher accumulation of oxidation products are met by higher cell turnover rates than in the ocean quahog.

The only symptoms of physiological change over age ever found in *A. islandica* were decreasing cell turnover rates in the heart muscle over a lifetime of 140 years. This may either indicate higher damage levels and possibly ongoing loss of functioning in the heart of aging clams, or, the opposite, lower rates of cell damage and a reduced need for cell renewal in the heart tissue of *A. islandica* over lifetime.

Basic physiological capacities of different *A. islandica* populations, measured at controlled laboratory conditions, could not explain considerable discrepancies in population specific MLSPs. For example, levels of tissue-specific antioxidant capacities and cell turnover rates were similarly high in individuals from the German Bight and from Iceland. Rather than genetic differences, the local impacts of environmental conditions on behavioral and physiological traits in the ocean quahog seem to be responsible for differences in population-specific MLSPs.

ZUSAMMENFASSUNG

Die Islandmuschel *Arctica islandica* ist nach dem heutigen Stand der Wissenschaft das langlebigste, nicht-koloniale Tier weltweit. Das bisher dokumentierte maximale individuelle Alter in einer isländischen Population liegt bei 405 Jahren. Deutlich niedrigere potentielle maximale Lebensspannen (MLSP) wurden in anderen *A. islandica*-Populationen, z. B. in der Kieler Bucht mit 30 Jahren und in der Deutschen Bucht mit 150 Jahren nachgewiesen, in deren Habitate stärkere jährliche Temperatur- und Salinitätsschwankungen gemessen wurden als in den Gewässern um Island. In der vorliegenden Arbeit wurden mögliche lebensverlängernde physiologische Mechanismen in *A. islandica* aus isländischen Gewässern und der Deutschen Bucht untersucht, sowie der Einfluss extrinsischer (z.B. Wassertemperatur und Nahrungsverfügbarkeit) und intrinsischer Faktoren (z.B. artspezifisches Verhalten) auf diese Strategien. Spezifisch wurde das Eingrabeverhalten im Feld und in Laborversuchen, die Herunter-Regulierung des Stoffwechsels (= metabolische Reduktion) sowie antioxidative und anaerobe Kapazitäten und der „Zell-Turnover" (= Apoptose- und Proliferationsraten) in unterschiedlichen Geweben der Islandmuschel untersucht. Apoptose- und Proliferationsraten wurden zudem in den Geweben der kurzlebigen, epibenthischen Kammuschel *Aequipecten opercularis* (MLSP = 8-10 Jahre) bestimmt, um mögliche Kapazitätsunterschiede in der Zell- und Gewebeerhaltung zwischen Muschelarten unterschiedlicher Lebensweisen und Lebenserwartungen zu zeigen.

Niedrige aerobe Stoffwechselraten sowie geringe oxidative Schädigung und konstante Schutzmechanismen in Zellen und Geweben scheinen den physiologischen Alterungsprozess zu verlangsamen und somit die außergewöhnlich lange Lebenserwartung von *A. islandica* entscheidend zu begünstigen. Im Vergleich zu der kurzlebigen *A. opercularis* wurden in *A. islandica* niedrigere Stoffwechselraten nachgewiesen. Zudem regulierte *A. islandica* den Sauerstoffpartialdruck (PO_2) des Mantelwassers auf weniger als 5 kPa herunter, einem Wert, bei dem die Bildung von reaktiven Sauerstoffverbindungen (ROS) in isoliertem Kiemengewebe zehnmal niedriger war als unter normoxen Bedingungen von 21 kPa.

Niedrigere Wassertemperaturen und eine geringere Nahrungsverfügbarkeit als im Sommer führten wahrscheinlich zu einem verstärkten Eingrabe-Verhalten und somit zu einer stärkeren Herunter-Regulierung des Stoffwechsels von *A. islandica* im Winter in den isländischen Gewässern. Diese energieeinsparenden Verhaltensweisen scheinen die Schlüsselparameter für die hohe Lebenserwartung dieser Muschelart zu sein. Das Signalmolekül Stickstoffmonoxid (NO) ist möglicherweise an der Regulierung der Stoffwechselraten in *A. islandica* während der Schließung der Schale und des Eingrabens beteiligt, indem NO bei niedrigem PO_2 die mitochondriale Cytochrom-c-Oxidase hemmt. Während eines Experiments mit isoliertem Kiemengewebe konnte die Respiration bei einem PO_2 von ≤10 kPa vollständig mit chemisch produziertem NO gehemmt werden. *Arctica islandica* erreicht den Zustand der metabolischen Reduktion jedoch nicht nur in eingegrabenem Zustand, sondern auch außerhalb des Sediments. So schlossen die Tiere selbstständig und ohne äußeren Reiz ihre Schale für einen Zeitraum von mehr als 24 h, und reduzierten den PO_2 des Mantelwassers auf 0 kPa. Unter diesen Bedingungen findet vermutlich keine ROS-Bildung mehr

statt. Die anaerobe Energiegewinnung setzt in *A. islandica* im Gegensatz zu anderen Muschelarten verzögert ein. Das anaerobe Stoffwechselprodukt Succinat akkumulierte erst nach 3,5 Tage in vollständig eingegrabenem Zustand oder nach einer Inkubationszeit von 3,5 Tagen unter Anoxie (0 kPa) im Adduktormuskel der Islandmuschel. Die Bildungsrate von ROS bleibt nicht nur während der Stoffwechselreduktion der Islandmuschel niedrig, sondern auch, wenn die Tiere an die Sedimentoberfläche zurückkehren, ihren Siphon öffnen und ihre Gewebe reoxygenieren. Demnach war die ROS-Bildungsrate isolierter Kiemengewebe nach Hypoxie-Reoxygenierungs–Experimenten (bei je 5 und 21 kPa) im Vergleich zu der Bildungsrate bei einem konstanten PO_2 von 21 kPa niedriger. Weder die gewebespezifische Aktivität der antioxidativen Enzyme Superoxiddismutase und Katalase, noch der Glutathiongehalt in den Geweben der Islandmuschel stieg während der Stoffwechselreduktion an.

Zusätzlich zu den niedrigen ROS-Bildungsraten im stoffwechselreduzierten Zustand, scheinen konstante Mechanismen der Zell-Erhaltung und –Erneuerung den physiologischen Altersprozess der Islandmuschel deutlich zu verzögern. Nicht nur die antioxidativen Schutzmechanismen in den Geweben von *A. islandica* sind bis zu einem maximal untersuchten Alter von 200 Jahren konstant. Auch die Zellerneuerungskapazitäten wie Apoptose und Proliferation blieben in den meisten Geweben der Tiere über das gesamte untersuchte Alter von 140 Jahren auf einem niedrigen, jedoch stabilen Level. Dementsprechend sind mit zunehmendem Alter die Akkumulationsraten von schädigenden Oxidationsprodukten (z.B. von Proteinen und Lipiden) in den Geweben der Islandmuschel niedriger als in der kurzlebigen Kammuschel *A. opercularis.*

Im Vergleich zu *A. islandica* ist die kurzlebige Kammuschel durch eine aktive Lebensweise, hohe Stoffwechselaktivitäten, absinkende Kapazitäten zellulärer Schutzmechanismen mit zunehmendem Alter und hohen Raten zellulärer oxidativer Schädigung gekennzeichnet. Dementsprechend wurden höhere gewebespezifische Apoptose- und Proliferationsraten in *A. opercularis* gemessen als in *A. islandica.*

Ausschließlich im Herzen von *A. islandica* nahmen sowohl Apoptose- als auch Proliferationsaktivität mit zunehmendem Alter ab. Bisher ist jedoch nicht bekannt, ob durch abnehmende Zellerneuerung die Zellschädigung im Herzen der Tiere mit ansteigendem Alter zunimmt, oder ob sinkende Apoptose- und Proliferationsaktivitäten nur eine Folge von verminderter Zellschädigungsrate sind und somit eine verminderte Zellerneuerungs-„Notwendigkeit“ mit zunehmendem Alter anzeigen.

In den unter konstanten Laborbedingungen gemessenen physiologischen Parametern konnten keine spezifischen Unterschiede festgestellt werden, welche die stark voneinander abweichenden populationsspezifischen Lebenserwartungen zwischen 150 Jahren (Deutsche Bucht) und 400 Jahren (Island) erklären könnten. Antioxidative Schutzmechanismen und Zellerneuerungskapazitäten unterschieden sich nicht zwischen den Individuen aus den beiden unterschiedlichen Populationen. Vermutlich werden die populationsspezifischen Unterschiede in der MLSP eher durch die Einwirkung lokaler Unterschiede in den Umweltbedingungen auf das Verhalten und die Physiologie von *A. islandica* hervorgerufen als durch genetische Unterschiede zwischen den Populationen.

1. INTRODUCTION

1.1. Bivalves as models in aging research

Bivalve mollusks have recently advanced to becoming new model species in aging research adding to the understanding of longevity with respect to biochemical, physiological and genetic mechanisms in short- and long-living bivalve species (Abele et al. 2009; Bodnar 2009; Philipp and Abele 2010; Ridgway and Richardson 2010). Bivalves are among the recordholders of long-living animal species. Maximum ages of more than 350 years have been reported for the ocean quahog *Arctica islandica* (Schöne et al. 2005a; Wanamaker et al. 2008), and the freshwater pearl mussel *Margaritifera margaritifera* has a maximum lifespan potential (MLSP) of 190 years (Ziuganov et al. 2000). In contrast, MLSPs in other bivalves such as the Bay scallop *Argopecten irradians irradians* or in surf clams are only 1 or 2 years (Powell and Cummins 1985; Estabrooks 2007).

In addition to their wide age-range, marine bivalves are known to perform a large variety of different lifestyles and adaptations to specific environmental conditions potentially affecting age and, thus, making them ideal model organisms for comparative age research (Pearse et al. 1987). For example, bivalves can be found firmly attached to the substratum (e.g. oysters and blue mussels, Barnes and Harrison 1997), swimming by jet propulsion (e.g. scallops, Jenkins et al. 2003), or burrowed more than 30 cm deep into the sediment (e.g. mud clams, Kvitek et al. 1988; Strasser 1999). These habitats may include temperature ranges from -1.8°C in polar waters (e.g. *Laternula eliptica* and *Adamussium colbecki*, Philipp 2005) to over 30°C in the tropics (e.g. oysters, Hawkins et al. 1998), salinities ranging from brackish waters (e.g. *Ostrea palmula* and *Polymesoda maritime*, Deaton 1981) to hypersaline environments (±60 PSU, e.g. *Fragum erratum*, Morton 2000), and pressures between 1 atm in the littoral zones (e.g. oysters and blue mussels, Bulnheim 1994) and up to ± 1000 atm in the deep sea (e.g. *Yoldiella jeffreysi*, Lightfoot et al. 1979).

In contrast to classical model organisms for aging research, such as the fruit fly *Drosphila melanogaster* or the nematode *Caenorhabditis elegans*, which are typically reared under controlled laboratory conditions for chronological age determination, the determination of age as well as the aging process in bivalves can be investigated in natural populations. In shells of bivalves which live in regions of strong seasonal fluctuations, annual growth increments are typically interspaced by narrow growth lines (= year rings) allowing for very accurate estimations of individual ages (Ropes et al. 1984) (Fig. 2.6A, B). Hereby, the growth lines are formed during the growth break in winter and are characterized by a different micro-structure and density in the calcareous material than in the growth increments, which are formed during the growth period (Jones 1980). The structural differences in the lines and the increments are related to seasonal changes of environmental conditions such as water temperature, salinity, oxygen- and feeding- conditions (Thompson et al. 1980; Brey und Mackensen 1997; Schöne et al. 2005b). For each bivalve species the annual pattern of these lines and increments can be validated on the basis of seasonal changes in stable oxygen isotope profiles (Witbaard et al. 1999) or through mark-recapture experiments in the field (Ropes 1988). In

each growth increment and line, the ratio of oxygen δ ^{18}O and δ ^{16}O or carbon δ ^{13}C and δ ^{12}C isotope pairs reproduce the pattern of environmental factors such as temperature and the content of seawater carbonate during the time of increment or line formation. For example, ^{18}O δ of seawater is inversely related to temperature and consequently it is the higher contents of this isotope which marks the winter growth lines of Antarctic mud clams (Brey and Mackensen 1997).

Accordingly, bivalves can be taken from their natural environment and individual ages can be inferred from the yearly growth lines or increments in the shell which, at the same time, archive changes in environmental conditions throughout the animals´ life time (Brey and Mackensen 1997; Epplé et al. 2006). In addition to the hard bodied shells, the soft body tissue of different bivalve species can be used to investigate physiological parameters in relation to age, and with respect to lifestyles and environmental conditions and details hereabout will be presented in the sections below.

1.2. Why is *Arctica islandica* an interesting model in aging research?

The ocean quahog *A. islandica* (Linnaeus 1767), also known as 'Iceland Cyprina', 'Black Quahog' or 'Mahogany Clam', is a non-selective suspension feeding bivalve. The name 'Mahogany Clam' refers to the golden brown periostracum, the outer organic shell layer typically found in young individuals (Ridgway and Richardson 2010) (Fig. 1.1A). In contrast, older individuals are called 'Black Quahog', which refers to the black color resulting from depositions of iron in the periostracum (Brey et al. 1990) (Fig. 1.1B). Maximum reported shell heights vary between 80 mm (Schöne et al. 2005a) and 90 mm (Witbaard 1997) to 100 mm (pers. observation). *Arctica islandica* generally resides directly beneath the sediment surface and maintains contact with the surrounding seawater through short siphons to take up oxygen and food (Winter 1969; Rowell and Chaisson 1983). The clams burrow in fine grained sediments (Rowell and Chaisson 1983) as well as in coarse-grained sand or gravel (Thorarinsdottir and Einarsson 1996) and can usually be found in water depths between 10 and 280 m (Kennish and Lutz 1995). *Arctica islandica* is widely distributed over the continental shelfs of both eastern North America and Europe covering a temperature gradient of 0°C to 19°C (Witbaard and Klein 1994) and a salinity gradient of 20 PSU to 35 PSU (publication V) (Fig. 1.2). In European waters the ocean quahog can be found around Iceland, Denmark, Norway and Sweden, in the North Sea, the Baltic Sea, in the English Channel as well as in the Irish Sea, the Barents Sea and the White Sea (Petersen 1915; Brey et al.1990; Rowell et al. 1990, Witbaard and Bergman 2003) (Fig. 1.2). In North Icelandic waters the species is a dominant member of the benthic infauna and occurs in mean densities of 14 ind. m^{-2} (Ragnarsson and Thorarinsdóttir 2002), whereas in the North Sea densities vary between 16 ind. m^{-2} and 0.1 ind. m^{-2} (Witbaard and Bergmann 2003). *Arctica islandica* is dioecious, and the larvae are planktotrophic. Reproduction usually commences at a shell size of 20 to 60 mm, corresponding to an age of 7 to 32 years in an Icelandic population (Thorarinsdóttir and Steingrimsson 2000).

The ocean quahog is not only the longest-lived bivalve worldwide, but also the longest-lived, non-colonial animal known to science (Wanamaker et al. 2008, Ridgway and Richardson 2010). Maximum individual ages of 375 years (Schöne et al. 2005a) and 405 years (Wanamaker et al. 2008) have been found in an Icelandic population, and of 150 years in a German Bight population (Witbaard and Klein 1994; Witbaard et al. 1999; Epplé et al. 2006). Interestingly though, maximum lifespan potential (MLSP) differs between populations, and low salinity populations of conspicuously shorter life expectancy have established in brackish waters of the subarctic White Sea with a MLSP of 53 years and of the temperate Baltic Sea with MLSP of 40 years (Begum et al. 2010).

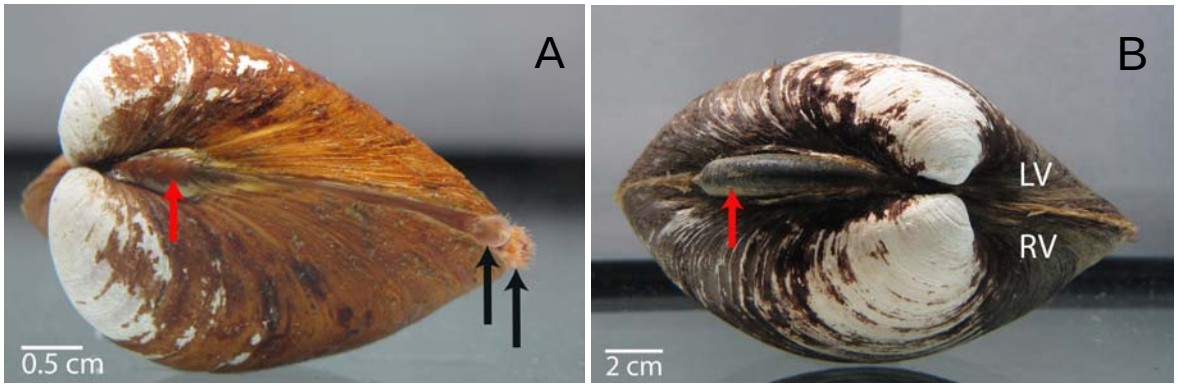

Fig. 1.1: (A) Young Iceland *Arctica islandica* with golden brown periostracum and open siphons (black arrows) and (B) older clam with black periostracum. The massive integument (red arrows) interconnects left shell valve (LV) and right shell valve (RV).

Recent studies on shell morphology show distinct variability among investigated *A. islandica* populations in the North East (NE) Atlantic Ocean including Iceland, the German Bight, the Norwegian Coast, the Kattegat, the White Sea and the Baltic Sea (Held et al. in prep.) (Fig. 1.2). However, the authors could not find any correlations of differences in shell morphology to geographical distances, nor were the patterns of shell morphology congruent to patterns of genetic variability. The study by Held and co-workers (in prep.) builds on earlier work by Dahlgren et al. (2000), who found only low genetic divergence in the investigated mitochondrial cytochrome *b* (cyt *b*) gene among 12 investigated populations in the NE and North West (NW) Atlantic Ocean, without any clear signs of an underlying geographical pattern (Held, pers. comm.). Although the highest genetic variability was found *within* and not *between* the 6 investigated NE Atlantic populations, recent analyses showed that the Iclandic and White Sea populations are clearly distinguishable from those of the Kattegat, German Bight, Baltic Sea and Norway (Held et al. in prep.). Yet, both morphometric and genetic data support the hypothesis of phenotypic plasticity in *A. islandica*, suggesting environmental adaptation and not genetic variability to cause the observed differences in shell morphology (Held et al. in prep). This may also hold true for differences in maximum lifespans of the populations from the NE Atlantic.

Its wide geographical occurrence at extremely diverse environmental conditions combined with its extreme longevity renders *A. islandica* a potential model organism for aging research (Ridgway and Richardson 2010) and for monitoring and modeling long-term environmental and ecological dynamics (Harding et al. 2008). The aging process and MLSPs of *A. islandica* from geographically separated populations seems to be controlled by extrinsic (environmental) rather than intrinsic (genetic) factors and subsequently gives rise to following questions: What are the major players in the physiological aging process of the ocean quahog? Are cellular processes modulated by environmental parameters? And can distinct differences in seawater temperature, salinity or food availability lead to variations in MLSPs of geographically separated *A. islandica* populations?

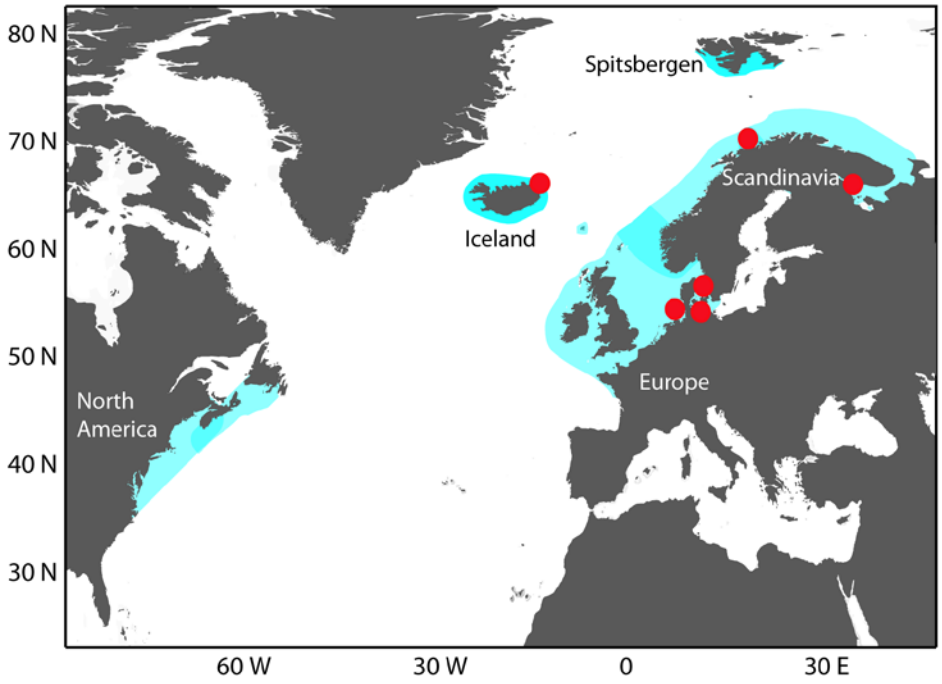

Fig. 1.2: Present-day distribution of *Arctica islandica* over the continental shelfs of both eastern North America and Europe including Scandinavian countries, Finland and Iceland (highlighted in blue), modified after Dahlgren et al. 2000. Physiological, morphometric and genetic studies were done in 6 different *A. islandica*-populations in the North East Atlantic including Iceland, German Bight, Norwegian Coast, Kattegat, White Sea and Baltic Sea (red circles). Map generated by online map creator at http://odv.awi.de.

1.3. What is aging?

The process of aging is regarded as a progressive functional decline, which can be observed in cells, tissues and organisms, and which results in an increased vulnerability to environmental challenge and a growing risk to suffer disease and to die (Wong 2001; Kirkwood 2005; Schmidt et al. 2005). The expression ‘senescence’ is mainly used to describe age-related changes in an organism that increase the mortality rate as a function of time and resulting in senility, the end stage of senescence, when mortality risk approaches 100 % (Finch 1990).

Today it is known that aging is a multifactorial determined and complex cellular process (Kirkwood 2005, Schmidt et al. 2005) and up to now more than 300 theories of aging have been formulated (Medvedev 1990), which can be separated into cellular and genetic theories. The latter propose that maximum life expectancy of a species is determined by genetic components, i.e. by single genes having major effects on longevity (Kirkwood 2005), or by the constant shortening of telomeres during each cell cycle which, having reached a critical length, inhibit cell division and initiate replicative senescence (Campisi et al. 2001; Shawi and Auexier 2008).

In contrast, cellular theories are related to physiological processes in cells and tissues which influence the organism´s lifespan and which can be modulated in ectotherms by environmental factors such as temperature, salinity or food supply. In 1956, Harman proposed the 'Free Radical Theory of Aging', which postulates that aging and degenerative diseases could be attributed to the destructive effect of reactive oxygen species (ROS) to cellular components. This theory completes the 'Rate of Living Theory' by Pearl (1928), suggesting an inverse correlation between standard metabolic rate (SMR) and lifespan. Both theories can be combined to the 'Free Radical-Rate of Living Theory', which propose a positive correlation between ROS formation and SMR (Fleming et al. 1981).

1.4. Physiological parameters involved in the aging process

Free radicals are highly reactive molecules and important oxidizing agents of biological substrates, containing one or more unpaired electrons (Halliwell and Gutteridge 2007). Apart from free radicals, many chemicals exist in biological systems which do not have radical properties, but are still important oxidizing agents such as hydrogen peroxide (H_2O_2), singlet oxygen or peroxynitrite (Halliwell and Gutteridge 2007).

ROS is a collective term describing pro-oxidants derived from oxygen which are generated continuously during aerobic metabolic processes, i.e. highly reactive superoxide anion radicals ($O_2^{-\cdot}$) and hydroxyl radicals ($^{\cdot}OH$) as well as H_2O_2. During mitochondrial respiration, ROS formation mainly occurs at complex I and III of the mitochondrial electron transport chain (Murphy 2009).

To avoid oxidative injury, cells are equipped with a variety of antioxidant enzymes, i.e. superoxide dismutase (SOD) and catalase (CAT) and with non-enzymatic antioxidant protection, i.e. glutathione, vitamin E and C or coenzyme Q (Finkel and Holbrock 2000; Balaban et al. 2005). In the literature, different definitions apply to the term 'antioxidant', but a common trait is that an antioxidant compound must *in vivo* significantly decrease the adverse effects of ROS (Constantini 2010). SOD is located in mitochondria, lysosomes, peroxisomes and in the cytoplasma, and catalyzes the reaction: $O_2^{-\cdot} + O_2^{-\cdot} + 2\ H^+ \rightarrow H_2O_2 + O_2$ (Fridovich 1978). Hydrogen peroxide is then catabolyzed by CAT, which is mainly found in the cytoplasm (Yu 1994): $2\ H_2O_2 \rightarrow 2\ HO_2 + O_2$ (Morgulis et al. 1926). The tripeptide glutathione, L-γ-glutamyl-L-cysteinyl-glycine (GSH), is the major low-

molecular-mass thiol compound (mol. wt 307) in cells of plants and animals (Meister and Anderson 1983; Schafer and Buettner 2001; Sies 1999). GSH is important both as substrate for antioxidant enzymes (e.g. glutathione peroxidase, glutathione S-transferase) and as a ROS scavenger of its own (Meister 1991). The majority of cellular GSH in vertebrates can be found in the cytosol, the principle location of GSH biosynthesis (Schafer and Buettner 2001), at concentrations of 1-11 mM (Smith et al. 1996). GSH can be oxidized by hydrogen peroxide, the highly reactive hydroxyl radical or organic peroxides to glutathione disulfide (GSSG) either non-enzymatically or via glutathione peroxidase catalysis (Stryer et al. 2003). The overall ratio of GSH/GSSG in a cell is usually greater than 100:1, and the redox couple GSSG/2GSH is used as indicator of changes in the redox environment and for oxidative stress in the cell (Schafer and Buettner 2001; Sies 1999).

Under certain conditions, cellular ROS production rates are higher than the rates of ROS decomposition or scavenging. Such a disturbance in the prooxidant / antioxidant balance has been described as oxidative stress (Sies 1985) and is reflected in changes of the cellular redox state (Klatt and Lamas 2000; Rebrin et al. 2003) and in oxidative damage of cellular proteins, DNA and membranes (Hermes-Lima 2004). Increasing damage to mitochondrial DNA, which is considered to be sensitive to ROS because of its proximity to the main source of oxidant generation, compromises mitochondrial function and integrity and finally leads to a higher release in ROS and lower ATP production (Finkel and Holbrock 2000). Due to this negative knock on effect, Balaban et al. (2005) coined this sequence the ‘vicious cycle’. Cellular ROS damage manifests as oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone, resulting in protein fragmentation and enzymatic inactivation (Berlett and Stadtman 1997). Further peroxidation of membrane polyunsaturated fatty acids can lead to deterioration of membranes, and modifications in proteins and DNA (e.g. base alterations, single-strand breaks, sister chromatid exchanges, DNA-protein cross-links), which result in altered cellular functions, cellular aging and finally cell death (Finkel and Holbrock 2000; Balaban et al. 2005).

1.5. Is the aging process in ectotherms related to reactive oxygen species formation?

ROS production rates are expected to be far lower in animals with lower oxygen consumption rates and living at lower environmental oxygen than in animals with high respiration rates and living at high environmental oxygen (Buttemer et al. 2010). When comparing related species of polar habitats to those of temperate habitats, polar ectotherms often exhibit a tendency for increased longevity (Brey 1991; Brey et al. 1995; Ziuganov et al. 2000; Philipp 2005; Philipp et al. 2006). This can partly be attributed to the temperature effects on metabolic rates and concomitantly on ROS formation rates and oxidative damage. In agreement, ROS formation rates at habitat temperatures and the decline in mitochondrial function with age are higher in short-lived temperate mud clams compared to longer-lived polar clams (Philipp et al. 2005a). In addition, total oxyradical scavenging capacities are reported to be higher in polar bivalves when compared to temperate species (Regoli et al. 2000; Camus et al. 2005).

Although a low ROS formation and high antioxidant capacities seem to be general characteristics of long-living species, this is not true for all marine invertebrates or bivalve species (Philipp et al. 2006; Buttemer et al. 2010). In contrast to mud burrowing clams, swimming scallops control mitochondrial ROS formation at extremely low levels (Philipp et al. 2006; reviewed in Buttemer et al. 2010). In spite of their low ROS levels, scallops – even when not falling prey to fish – never reach the outstandingly long lifespan of mud clams. Thus, there must be other mechanisms such as high energy investment into fast growth, mobility and reproduction and less into cellular maintenance that cause the early death in scallops.

Long-lived marine organisms such as the ocean quahog *A. islandica* may have developed several behavioral and physiological mechanisms to maintain cells and tissues vital as long as possible throughout their life time. Indeed, tissue-specific antioxidant capacities were higher in *A. islandica* compared to shorter-lived bivalves and constant over 200 years of life time (Abele et al. 2008). Especially in the gills, fourfold higher CAT-activity, tenfold higher SOD-activity and twofold higher contents in total glutathione (GSH + 2 x GSSG) were found in *A. islandica* compared to other mud-burrowing clams or scallops (Abele et al. 2008). But high antioxidant protection alone cannot explain extraordinarily long life expectancies in *A. islandica*, which is 10-100 times longer than that of most other bivalve species (Powell and Cummins 1985; Philipp 2005; Estabrooks 2007). Primarily the combination of high cellular defense and repair mechanisms, efficient mechanisms to remove and replace damaged cells as well as slowing of metabolism (=metabolic rate depression) and high resistance to environmental stressful conditions (e.g. chances in temperature, salinity, oxygen content or food availability) may be responsible for the lack of senescence in extraordinarily long-lived species such as the ocean quahog.

1.6. Cellular maintenance and longevity

While high antioxidant protection helps to prevent oxidative injury in cells and tissues, proliferation (cell division) and apoptosis (programmed cell death) are essential processes in multicellular organisms to eliminate and rejuvenate tissues, which are damaged, infected or senescent (Sokolova 2009). For extraordinarily long-lived species it is believed that they make great investments into cellular maintenance and repair (Kirkwood 2005). Martinez (1998) found the capacity for constant cell renewal in tissues by continuous production and displacement of epithelial and interstitial cells to be the main reason for the potential immortality of the freshwater polyp *Hydra vulgaris*. Apoptosis is highly conserved in evolution and plays a critical role in cellular and tissue homeostasis, embryonic development and immune defense in vertebrates and invertebrates (reviewed in Sokolova 2009). In contrast to necrosis (defined as ‘accidental cell death’), it is a highly regulated energy consuming process in which intracellular compounds are fractionized and disposed of in an orderly manner without the induction of inflammation. Programmed cell death can be triggered by environmental factors such as thermal and salinity stress, UV-radiation and pollution (Wadskog et al. 2004; Wilczek 2005; Richier et al. 2006). On the molecular base, apoptosis is triggered either by

intrinsic (mitochondrial) or extrinsic (extracellular environmental) signals which both initiate a cascade of caspase activation, a family of proteases involved in the apoptotic process (Feig and Peter 2007) (Fig. 1.3). The cell can sense internal problems that call for the activation of the intrinsic apoptotic pathway triggered by toxic insults or stress factors such as oxidative damage, genome instability, viral infection, UV-radiation and/or growth factor withdrawal (Feig and Peter 2007; Circu and Aw 2008). In vertebrates a key initial event of the intrinsic pathway is the release of cytochrome-*c* from the mitochondrial intermembrane space into the cytosol, where it binds to other molecules to form a complex called apoptosome which in turn activates the caspase-cascade (Feig and Peter 2007; Circu and Aw 2008) (Fig. 1.3). The extrinsic apoptotic pathway (death receptor pathway) is initiated by binding of specific protein ligands to death receptors on the cell surface (Sokolova 2009). Initiator caspases then cleave and activate effector caspases which, in turn, act upon intracellular targets (e.g. cytoskeleton proteins, mitochondria or chromatin) and lead to cell decomposition (Fig. 1.3).

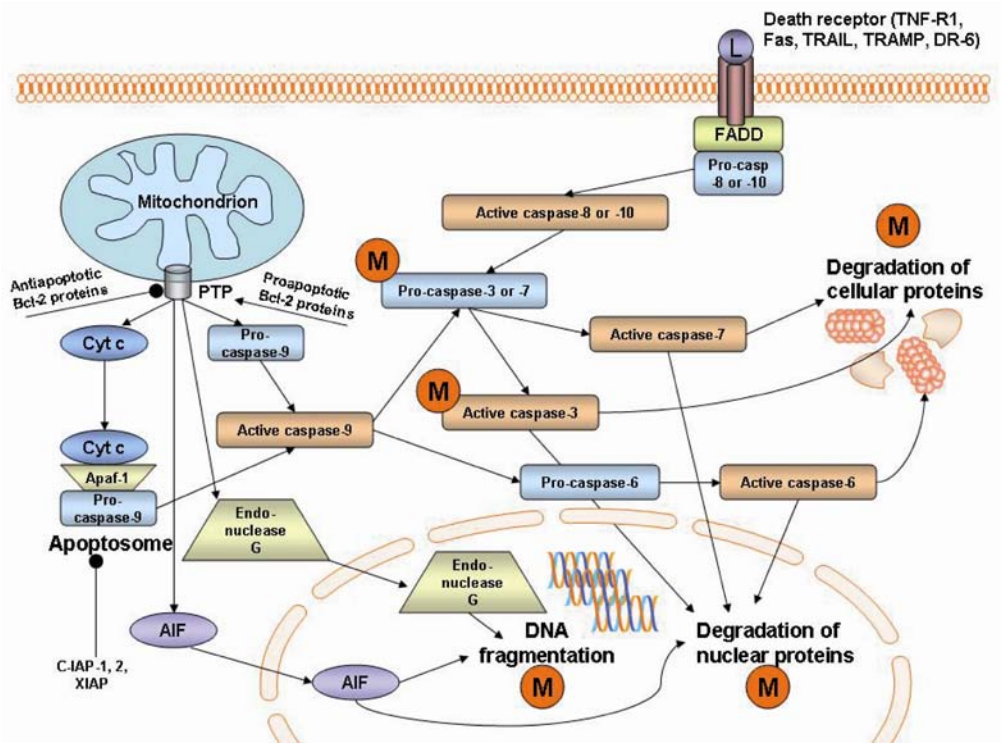

Fig. 1.3: Major apoptotic signaling and execution pathways, taken from Sokolova (2009). Extrinsic (death receptor-activated) pathways are shown on the right of the diagram, and intrinsic (mitochondrial) pathways are shown on the left of the diagram. Red circles with the letter 'M' indicate pathways that have been demonstrated in mollusks, PTP = permeability transition pore, Cyt c = cytochrome c, Pro-casp = procaspase, FADD = Fas-associated death domain, Apaf-1 = Apoptotic peptidase activating factor 1,; AIF = apoptosis inducing factor, IAP = inhibitor of apoptosis family of proteins.

Dead cells are replaced during cell proliferation, which is defined as the increase in cell number resulting from completion of the cell cycle (Pardee 1989). The process of proliferation is tightly controlled, e.g. by concentration of regulatory proteins and/or stability and concentration of

enzymes involved in the cell cycle such as thymidine kinase, DNA polymerase or ribonucleotide reductase. Cell division is determined by different extracellular factors (e.g. growth factors, oncogenes, inhibitors) and intracellular events during the G_1-phase (e.g. movement of DNA-catalyzing enzymes into the nucleus) in vertebrates and invertebrates (Raftos et al. 1991; Pardee 1989).

1.7. Metabolic rate depression and longevity

Under harsh environmental conditions, which limit resources or access to resources, many invertebrates enhance their chances of survival by entering a dormant or quiescent state of hibernation, torpor, estivation and diapauses (reviewed in Stuart and Brown 2006). Dormancy is characterized by starvation and metabolic rate depression (MRD), which describes a concordant decline in energy supply and energy demand (Stuart and Brown 2006; Moullac et al. 2007). A substantial part in energy saving comes from the suppression of physiological activities such as reduced muscle movement, heart beat and respiration rates and, because animals are in starvation during these periods, energetic costs of digestion, nutrient absorption, and peristalsis are curtailed (Storey and Storey 2004). Major energy saving during dormancy and MRD comes from down-regulation of multiple cellular processes such as reduced mitochondrial respiration (Guppy et al. 2000), suppression of membrane channels and ATP-dependant ion pumps (Bickler et al. 2001) and of the rates of synthesis versus degradation of macromolecules including DNA, mRNA, proteins and membrane phospholipids (Story and Storey 2004). Estivating snails are able to reduce their metabolic rates by about 70-85 % (Pedler et al. 1996; Michaelidis 2002) and their protein translation by 80 % within two days of estivation (Ramnanan et al. 2009). The brine shrimp *Artemia fransciscana* is even capable of entering an encysted diapause state in which metabolic rate is reduced to essentially zero (Clegg 1997).

While MRD in several invertebrate species is exclusively linked to unfavorable environmental conditions, this is not the case in the infaunal ocean quahog *A. islandica.* This species is capable of self-induced burrowing and metabolic rate depression (MRD). Hence, periods where the clams are respiring at the sediment surface are interspersed with periods where the animals are burrowed several centimeters deep in the sediment. During periods of burrowing, which last between 1 and 7 days, the internal oxygen partial pressure (PO_2) declines to 0 kPa, which causes a state of MRD and finally leads to anaerobiosis (Taylor 1976). The heart-beat of *A. islandica* decreases from 10 beats min^{-1} to 1-2 beats min^{-1} after 24 h of burrowing (Taylor 1976), and after 50 days of anoxia incubation the metabolic rates are reduced to 1 % of the aerobic values (Oeschger 1990).Intermittent burrowing and self-induction of MRD could be life-prolonging strategies in *A. islandica* due to the frequent and repeated slow-down of aerobic metabolism and the simultaneous down-regulation of energy consuming processes and ROS production rates. To date, however, studies examining the effect of MRD on rates of aging in bivalves are still scarce.

In the hypometabolic dauer larvae of *C. elegans*, MRD is associated with an eightfold prolongation in life expectancy (Riddle and Albert 1997). Similarly, encysted *A. fransciscana* in the MRD status remain alive for as long as 332 years, while active animals live less than 6 months (Hairstone et al. 1995). In the animals with diapauses-capacities, changes in the physical environment activate highly conserved biochemical regulatory mechanisms that control MRD (Storey and Storey 2004, Melvin and Andrews 2009). In contrast, in *A. islandica*, neither extrinsic (e.g. changes in seawater temperature, salinity or food availability) nor intrinsic mechanisms could be linked to cellular regulatory mechanisms in order to plausibly explain the species-specific burrowing behavior and metabolic suppression (Ridgway and Richardson 2010). A metabolic concept in which the signaling molecule nitric oxide (NO) may take over a role in MRD-induction in the ocean quahog during burrowing and shell-closure, is most likely of paramount importance.

1.8. Possible role of nitric oxide in metabolic rate depression

NO is a free radical and signalling molecule of potential importance which is evolutionary and functionally conserved in vertebrates and invertebrates (Palumbo 2005). NO can be generated in invertebrate cells and tissues either enzymatically (Moroz and Gilette 1995; Gladwin et al. 2005) or non-enzymatically (Zweier et al. 1999), but also by bacteria in the seawater or sediments (Strous et al. 1999). In mollusks, NO is a potent messenger molecule within the nervous systems (reviewed in Moroz and Gillette 1995), and NO produced by hemocytes leads to the killing of invading bacteria and pathogens (Tafalla et al. 2003). In vertebrate cells, NO has been recognized as a potent mitochondrial regulatory metabolite by directly inhibiting cytochrome-*c*-oxidase, the terminal electron acceptor of the mitochondrial electron transport chain (Boveris et al. 2000; Turrens 2003). NO binding to the enzyme is reversible, competitive with O_2 and, therefore, dependent on the cellular oxygen concentration (Boveris et al. 2000) and results in the inhibition of the entire mitochondrial respiration (Cooper 2002).

Moroz and Gillette (1995) reported NO concentrations in mollusk hemolymph between 30 and 300 nM, and attributed these to the rather low mean PO_2 measured in molluskan hemolymph: 20-40 torr (2.5-5 kPa) in non-specified marine mollusks and 5-12 torr (0.7-1.6 kPa) in fresh water snails. Equally low values are reported for marine bivalves, including *A. islandica*, which actively down-regulate mean PO_2 in the mantle cavity water to < 5 kPa also at full oxygenation (21 kPa) of the seawater (Abele et al. 2010). During prolonged burrowing phases and shell-closure, hemolymph and mantle cavity water PO_2 declines to even lower levels (Taylor 1976). Thus, NO concentrations may be stabilized in the hemolymph of *A. islandica*, which could then diffuse into the cells and tissues and reduce mitochondrial respiration by inhibiting cytochrome-*c*-oxidase.

1.9. Metabolic rate depression, anaerobiosis and recovery from anoxia

At a certain threshold during prolonged hypoxic or beginning anoxic conditions, which is called critical oxygen point (P_{crit}), hypoxia-tolerant invertebrates are no longer able to transfer sufficient

amounts of oxygen to tissues and cells to meet energy requirements aerobically (Pörtner and Grieshaber 1993). Thus, they gradually switch from aerobic to anaerobic energy production.

In marine invertebrates functional and environmental anaerobiosis are distinguished (Grieshaber et al. 1994). In bivalve mollusks, the former can be brought about by physiological activity, for instance, enhanced muscular activity during burrowing of mud clams (Schiedek and Zebe 1987), or swimming-escape reactions of scallops (Philipp et al. 2008). The latter is due to the limitation of the amount of oxygen in the water or sedimentary environment. In the German Bight additive effects of anthropogenic organic discharge together with reported thermal and saline stratifications and steadily increasing water temperatures induced severe hypoxia of bottom water masses during several summer periods (Grieshaber et al. 1994; Wiltshire and Manly 2004). Most mollusks are highly tolerant to hypoxia and anoxia (Moullac et al. 2007). But in contrast to other bivalve species, *A. islandica* is able to survive more than 50 days of experimental anoxia (Oeschger 1990), and further, self-induces internal hypoxia and anoxia during prolonged burrowing periods and shell-closure (Taylor 1976). When energy requirements in the cell exceed both aerobic energy production and ATP production by transphosphorylation of phosphorylated guanidinium compounds (e.g. phosphor-L-arginine, phosphocreatin or phosphoglycocyamine) which serve as rapid available stores of phosphate-bond energy, the metabolism switches to anaerobiosis. Main anaerobic energy providing pathways in the cytosol of invertebrate cells are based on anaerobic glycolysis and the reductive condensation of pyruvate and different amino acids (Grieshaber et al. 1994). The latter can be catalyzed by 5 different dehydrogenases, i.e. lactate, octopine, alanopine, strombine and tauropin dehydrogenase. In addition to lactate, which is the main end-product of functional anaerobiosis in vertebrate tissues, opines such as octopine, alanopine, strombine and tauropin can also be formed during physiological activity in invertebrates (Ulrich 1990) (Fig. 1.4). During environmental anaerobiosis, energy is provided by more efficient anaerobic pathways located in the mitochondrial compartments, with the main end-products succinate, propionate and acetate (Ulrich 1990, Grieshaber et al. 1994) (Fig. 1.4).

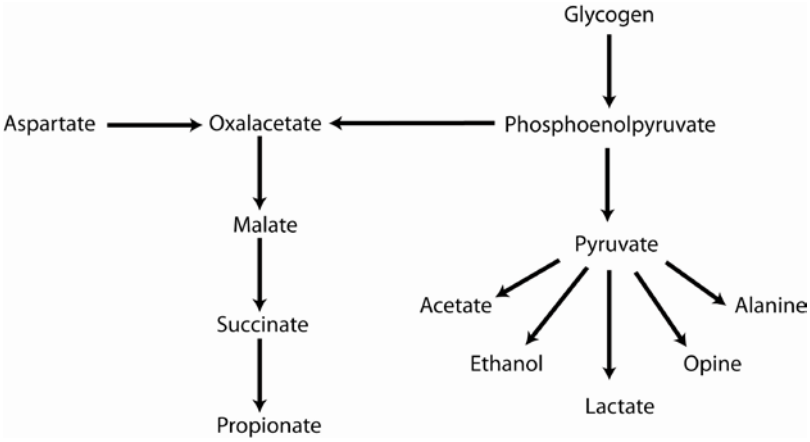

Fig. 1.4: Endproducts of the anaerobic metabolism in animals, modified after Ulrich 1990.

Whereas MRD itself may reduce energy expenditures and ROS production, the recovery from anoxia during surfacing of the ocean quahog may increase oxygen expenditures due to increased energy demands for disposal of anaerobic metabolites and recharging the phosphagen and ATP pools, as observed in several invertebrate species (Hereid 1980; Ellington 1983). This in turn could increase mitochondrial ROS formation rates and, correspondingly, higher levels of free radical damage during awakening and tissue reoxygenation, which are reported for estivating and hibernating invertebrates (Ramos-Vasconcelos and Hermes-Lima 2003). Lipid peroxidation was increased by 25 % in the hepatopancreas of the land snail *Otala lacteal* after 20 min of arousal from dormancy (Hermes-Lima and Storey 1995).

Although anaerobic capacities are known to be high in tissues of hypoxia-tolerant *A. islandica* (Taylor 1976; Livingstone et al. 1983; Oeschger 1990; Oeschger and Storey 1993), it is yet unresolved, at which PO_2 aerobic metabolic rates are simply down-regulated and at which PO_2 anaerobiosis starts (= P_{crit}). It is further unclear which amounts of metabolites accumulate during self-induced MRD in tissues of *A. islandica*, and whether or not oxygen consumption and ROS production increase after anoxia-reoxygenation events during surfacing.

1.10. Aims of the thesis

The aim of this thesis was to identify possible life-prolonging strategies in the extraordinarily long-living ocean quahog *A. islandica* and to examine the modulating effects of extrinsic factors (e.g. seawater temperature, food availability) and intrinsic factors (e.g. species-specific behavior) on these strategies. Tissue-specific cell turnover rates, tissue-specific antioxidant and anaerobic capacities, as well as burrowing behavior and MRD were investigated in *A. islandica* from Iceland and the German Bight with respect to different local temperature regimes. Induction of metabolic rate depression was examined in field and laboratory studies, and the possible role of NO as a signaling molecule in this process was tested. For an inter-species comparison of physiological parameters the mud-burrowing clam *A. islandica* and the swimming scallop *A. opercularis* with highly different life expectancies where used.
Specific questions were:

(i) Has the ocean quahog *A. islandica* developed high cellular defense or repair mechanisms or efficient mechanisms to remove and replace damaged cells? Are these mechanisms constant over age and, thus, responsible for a lack of senescence over 200 years of lifetime? Do bivalves with differing lifespans and lifestyles, or populations of the same species yet living under different environmental conditions, follow a common pattern in cellular maintenance and repair?

(ii) Are burrowing and self-induced MRD life-prolonging strategies in *A. islandica*? Are frequencies of MRD in different populations of the ocean quahog modulated by environmental parameters such as seawater temperature, salinity and/or food availability which may lead to longer MLSP in Iceland *A. islandica*? Is MRD in *A. islandica* accompanied by oxidative injury during surfacing and tissue reoxygenation?

(iii) Is hypoxia and anoxia-tolerance in *A. islandica* primarily based on the down-regulation of aerobic metabolic rates and on shifting the onset of anaerobiosis to extremely low internal PO_2 levels? Is there a low tissue PO_2 threshold in hypoxia tolerant ocean quahog down to which aerobic energy metabolism can be maintained by economizing energy expenditures and below which the animals need to switch to anaerobiosis?

(iv) Is the signaling molecule NO produced by hemocyte cells of the ocean quahog? And is NO involved in the onset of MRD in *A. islandica* by inhibiting mitochondrial respiration?

2. MATERIALS AND METHODS – A GENERAL OVERVIEW

All studies of the thesis were conducted at the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven and at the University of Bremen, Germany. In laboratory and field experiments, investigations of the cell turnover, the metabolism, the burrowing behavior and both self-induced and forced MRD and anaerobiosis in live *A. islandica* were conducted. For comparison, specimens from a warm-adapted population of the German Bight (North Sea) and specimens from a cold-adapted Icelandic population were chosen. For inter-species comparison, cell turnover rates were investigated in individuals of the short-lived scallop *Aequipecten opercularis*. A brief summary of the biology and ecology of this species is given below.

After finalization of all laboratory experiments with the live bivalves, these individuals were subsequently dissected and tissue samples of all individuals were either directly analyzed or snap-frozen in liquid nitrogen for further physiological analysis.

The methods to all experimental work as well as to the measuring of different physiological parameters are explicitly described in the publications I-III (chapter 3) and in the additional results (chapter 5). For this reason, they are only briefly summarized here.

2.1. Species under study and sampling locations

Arctica islandica: a long-living, burrowing benthic mud clam (see chapter 1.2 for species description)

In May and August 2008, individuals of *A. islandica* were sampled with trawl nets from the 'Tiefe Rinne', a 40-45 m deep depression of the seabed south of Helgoland in the German Bight (54°09.05'N, 07°52.06'E), and from 8-15 m deep sandy grounds northeast of Iceland (66°01.44'N, 14°50.91'W), respectively (see also chapter 1, Fig. 1.2). In the sampling areas, seasonal seawater temperatures range from 4-19°C in the German Bight (http://www.bsh.de/de/Meeresdaten/ Beobachtungen/MURSYS-Umweltreportsystem/index.jsp) and from 2-10°C at Iceland (http://www.hafro.is/Sjora).

Aequipecten opercularis: a short-lived, swimming epibenthic scallop

Aequipecten opercularis (Linnaeus, 1758, Fig. 2.1), also referred to as queen scallop, has a wide geographical distribution on the European continental shelf reaching from Northern Norway to the Mediterranean Sea (Waller 1991). These filter feeding scallops experience seasonal seawater temperatures of 6-24 °C (Ansell et al. 1991) and can be found in water depths of up to 1,000 m (Dell 1972). The maximum reported lifespan of *A. opercularis* is 8-10 years with a maximum shell height of about 90 mm (Philipp et al. 2006). Queen scallops are hermaphrodites shedding planktotrophic larvae during spawning seasons (Shumway and Parsons 2006). *Aequipecten opercularis* move through jet propulsion, reaching speeds of up to 30 cm s^{-1} (Moore and Trueman 1971). An

escape reflex is elicited when the distance to potential approaching predators or hazards, such as fishes, scuba-divers or fishing gear, falls below 1.5 m (Chapman 1981).

In August 2008, individuals of *A. opercularis* were collected with a toothed-dredge in the English Channel (48°43.07´N, 03°59.02´E) at about 60 m water depth. Similar to the temperature ranges in the German Bight, the seasonal temperatures in the English Channel fluctuate between 6 and 18°C (Robin and Denis 1999).

Fig 2.1: The scallop *Aequipecten opercularis*, Photo by E. E. R. Philipp.

2.2. Experimental studies

2.2.1. Incubation experiment for the determination of cell-turnover rates

To compare cell turnover rates in tissues of the mud clam *A. islandica* and the scallop *A. opercularis*, proliferation rates and apoptosis intensities were measured in different tissues of both bivalve species (publication I). *Arctica islandica* and *A. opercularis* and were maintained individually in 3-l flasks filled with natural seawater incubated with 5-Bromo-2-deoxyuridine and 5-Fluoro-2-deoxyuridine, which were taken up by the bivalves with the inhaled medium. Control bivalves were held individually in seawater without proliferation reagents. Bivalves were dissected and blocks of the target tissues transferred to cryovials and snap frozen in liquid nitrogen for both proliferation and apoptosis measurements. The shells of all bivalves were cleaned and numbered for individual age determination.

2.2.2. Field and laboratory studies on the burrowing behavior and self-induced metabolic rate depression in *Arctica islandica*

Burrowing experiment in the field

The seasonal burrowing behavior of *A. islandica* was investigated in Eyjafjördur, North Iceland (publication III). In June 2003 and in February 2004, the burrowing depths of individual clams were determined *in situ* within defined seabed-areas at the sampling site by the diving team of Gudrun Thorárinsdottir (Marine Research Institute Reykjavik) in following manner: when the openings of the short siphons of *A. islandica* were visible at the sediment surface (Fig. 2.2), the burrowing depth was considered 0. In deeper burrowed clams, where siphons were invisible, the depth of

burial was measured to the nearest 5 mm as the distance from the seafloor surface to the uppermost part of the clam.

Laboratory burrowing experiment

Arctica islandica were maintained in flow-through seawater tanks at 33 PSU and 10°C containing 20 cm of sediment. Burrowing-depth was recorded daily for every bivalve (see method above) and the siphon-status of each animal (i.e. opened versus closed) was documented individually using a video camera (publication III). Bivalves, which were burrowed in the sediment deeper the 3 cm for a continuous period of 3.5 days were identified as metabolic rate depression (MRD) animals and sorted out. Bivalves with their siphons opened at the sediment surface for a continuous period of 3.5 days were identified as normoxic control animals and sorted out as well. Mantle cavity water of MDR and normoxic bivalves was sampled and tissue samples were taken and snap frozen in liquid nitrogen for further biochemical analysis.

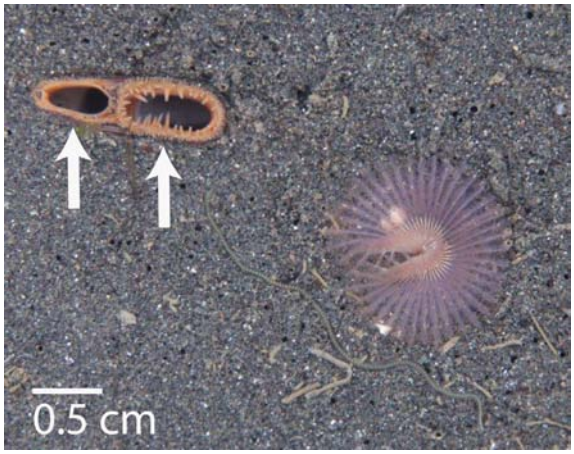

Fig. 2.2: Visible openings of the paired siphon (white arrows) of *Arctica islandica* burrowed directly beneath the sediment surface nearby Eyjafjördur, North Iceland. The inhalant siphon (right arrow) is bigger than the exhalant siphon (left arrow) and is surrounded by numerous large tentacles. Photo by Arnoddur Erlendsson.

Measurements of mantle cavity water PO_2

In order to investigate whether *A. islandica* self-induces MRD and voluntarily lowers internal PO_2 at normoxic seawater conditions and when deprived of their sedimentary retreat, mantle cavity water PO_2 was measured with implanted needle optodes in live specimens in cooperation with Natalie Fischer (University of Bremen) (publication III, Fig. 2.3). The siphon-status of each experimental clam was documented using a video camera. Tissues of bivalves that maintained zero PO_2 for ≥ 24h were dissected and snap-frozen in liquid nitrogen as MRD-samples, and bivalves that did not maintain constant 0 % oxygen as normoxic samples. Tissue samples were stored in liquid nitrogen until further biochemical investigations.

In order to test for physiological adjustments regarding the status (MRD versus normoxic) of *A. Islandica* from the laboratory burrowing experiments and the PO_2-measurements, the frozen tissue samples were analyzed for antioxidant enzyme activities, total adenylates and citrate synthase (CS) activity, accumulation of the anaerobic metabolite succinate as well as for hemocyte-NO-production which was indirectly determined by measuring the nitrite and nitrate-concentration with the Griess Assay (publication III, chapter 5.1).

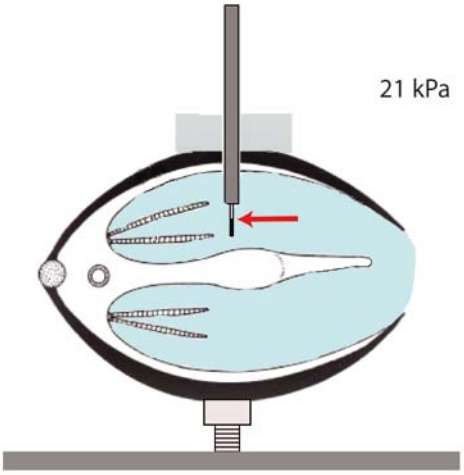

Fig. 2.3: Schematic overview of a fixed *Arctica islandica* in a laboratory aquarium at normoxic seawater (PO_2 = 21 kPa), modified after Brusca and Brusca (1990). The implanted needle optode (red arrow) was inserted through a drilling hole in the shell and extended into the mantle cavity water (light blue) of the ocean quahog to measure the internal PO_2.

2.2.3. Laboratory study of forced metabolic rate depression

Incubation experiments at three different environmental PO_2

In order to distinguish between metabolic and physiological responses to low oxygen conditions versus full anoxia and normoxic control conditions in mantle, gill, adductor muscle and hemocytes of the ocean quahog, specimens were maintained for 3.5 days under normoxia (21 kPa O_2), hypoxia (2 kPa O_2) or anoxia (0 kPa O_2) (publication II). Herefore, *A. islandica* were maintained individually in 3-l glass jars filled with natural seawater (33 PSU) at 10°C and exposed to each of the three treatments (Fig. 2.4). At the end of the incubation experiments hemolymph was taken from the adductor muscle of each individual and tissues were excised to determine the following parameters of the anaerobic metabolism: octopine dehydrogenase (ODH) and lactate dehydrogenase (LDH) activity as well as the accumulation of octopine, lactate and succinate (publication II). Tissue and hemocyte samples were analyzed for content of the ROS-scavenger glutathione (publication II) and for nitrite and nitrate-contents (chapter 5.1).

Fig. 2.4: *Arctica islandica* individually maintained in 3-l glass flasks filled with natural seawater incubated at normoxia, hypoxia or anoxia.

2.2.4. Measurement of ROS-formation in isolated gill tissue

Arctica islandica is supposed to experience high fluctuations of tissue oxygenation during burrowing and surfacing. Thus, in cooperation with Wiebke Wessels (University of Bremen), ROS-formation rates were determined in isolated gill filaments of *A. islandica* preincubated at normoxic (21 kPa O_2) and hypoxic (5 kPa O_2) conditions, and after hypoxia-reoxygenation, using the ROS-sensitive fluorescent dye dihydroethidium (publication III).

2.2.5. Measurement of aerobic metabolic rates

Measurement of standard metabolic rates

Respiration rates of *A. islandica* were measured in multi-channel intermittent flow systems (publication I, III, IV) as described in Heilmayer and Brey (2003). Oxygen consumption of actively respiring bivalves was individually recorded. Experimental animals that did not consume oxygen during 24 h in the respiration system were recorded and substituted by other test specimens. Following each measurement, the clams were dissected to obtain ash free dry mass and to calculate standard metabolic rate.

Measurement of gill respiration rate

Gill respiration rates of *A. islandica* were measured with oxygen needle optodes (publication II, chapter 5.1) (Fig. 2.5). Gill respiration rate was measured in thermostated respiration chambers filled with respiration-buffer (Fig. 2.5) and adjusted to different PO_2s between 21 kPa and 2 kPa. After the measurements, the gill samples were weighed and the respiration rates were calculated.

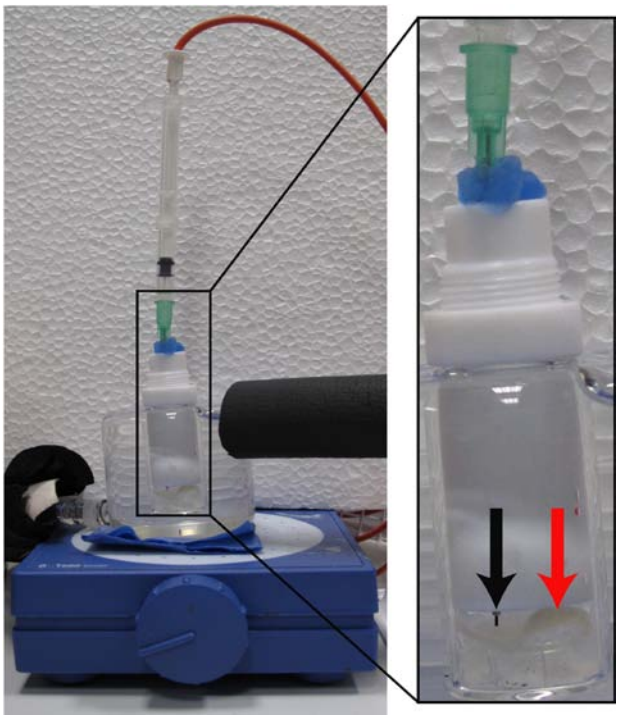

Fig. 2.5: Gill respiration measurement in *Arctica islandica* with an oxygen needle optode (black arrow), which is introduced into the cooled respiration chamber through a hole in the stopper. The gill sample (red arrow) is surrounded by 1.1 ml of respiration-buffer in the chamber.

2.2.6. Investigation of nitric oxide formation and of its possible role as modulator of cellular respiration

Measurement of nitric oxide production

Mantle cavity water from *A. islandica* was taken with a sterile needle and a sterile 10-ml syringe. The sample was centrifuged, the supernatant discarded and the pellet containing the hemocytes was diluted in filtered seawater to measure NO production using a NO-electrode (chapter 5.1).

Inhibition of gill respiration

In order to determine, if NO reduces mitochondrial respiration in *A. islandica*, gill respiration rates were measured in two parallel chambers at different PO_2s between 16 kPa and 2 kPa, using two gill pieces of each experimental animal (chapter 5.1). In the first chamber, only respiration rates were determined (see method above). In the second chamber gill respiration was inhibited with the NO-donor sperminNONOate. After completely inhibited respiration, the inhibition was neutralized by the NO-scavenger oxyhemoglobin.

2.3. Biochemical Assays

2.3.1. Proliferation rates

Proliferation rates in the tissues of *A. islandica* were determined after Moore et al. (1994) (publication I). Cryosections of all tissue samples were stained using an Amersham cell proliferation kit (GE Healthcare, Buckinghamshire, UK) to visualize dividing nuclei in the tissues. A second cryosection of each sample was stained in hematoxylin and eosin-phloxin to visualize all nuclei in the tissue section and to calculate the percentage of cell proliferation per day.

2.3.2. Apopotosis intensities

Apoptosis intensities were determined in tissue homogenates of *A. islandica* as caspase-3 and -7 activities using a Caspase-Glo 3/7 Assay kit (Promega, Madison, USA) after Liu et al. (2004) (publication I). Samples were analyzed using a Multilabel Reader LB 941 TriStar (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany) and apoptosis intensities were calculated.

2.3.3. Mitochondrial enzyme activity

Citrate synthase activity was measured in tissue homogenates of *A. islandica* after Sidell et al. (1987) at 20°C (publication III).

2.3.4. Adenylate concentrations and energy charge

The contents of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in tissue homogenates of *A. islandica* were determined after Lazzarino et al. (2003), using high performance liquid chromatography (publication III). The total amount of adenylates (= ATP + ADP + AMP) was added up and the energy charge was calculated.

2.3.5. Antioxidant defense parameters

Superoxide dismutase and catalase activities and the specific glutathione content (GSx = GSH + 2 x GSSG) were measured in homogenates of tissues and hemocyte cells of *A. islandica* (publication II and III). The glutathione content was measured as described by Dringen and Hamprecht (1996) in the microplate reader according to the colorimetric method described by Tietze (1969). The activity of antioxidant enzymes SOD and CAT were determined photometrically at 20°C according to Livingstone et al. (1992) and Aebi (1984).

2.3.6. Anaerobic enzyme activity and accumulation of anaerobic metabolites

Lactate dehydrogenase and octopine dehydrogenase activities were measured in tissue homogenates of *A. islandica* using a mircroplate reader according to Livingstone et al. (1990) (publication II). The accumulation of anaerobic metabolites lactate, octopine and succinate was determined in the tissue homogenates and the hemolymph of *A. islandica* and in the seawater in which the

bivalves were incubated (publication II and III). Octopine and lactate content were determined in the microplate reader after Luisi et al. (1975) and Schmidt and Dringen (2009), respectively. The succinate content was determined after Michal et al. (1976) using a succinic acid Assay kit.

2.3.7. Nitrite and nitrate content

Nitrite and nitrite contents in mantle cavity water and hemolymph of *A. islandica* and in the seawater in which the bivalves were incubated were measured with the Griess-assay according to Misko et al. (1993) and Verdon et al. (1994) (chapter 5.1).

2.4. Individual age determination

In order to relate cell turnover rates to age, individual ages of *A. islandica* and *A. opercularis* were determined (publication I). Individual ages of *A. islandica* were determined from internal shell growth bands, following the procedure of Strahl et al. (2007). Each valve was sectioned along the line of strongest shell growth and annual growth increments in the umbo (Fig. 2.6 A) and along the outer shell side (Fig. 2.6 B) were counted under a reflected-light stereomicroscope (Olympus SZX12, Germany).

In *A. opercularis,* the individual age was inferred from shell height and the 'Von Bertalanffy growth model' (VBGM) established by Heilmayer et al. (2004b) and based on height-at-age-data of the same scallop population from the English Channel which was sampled in the present study.

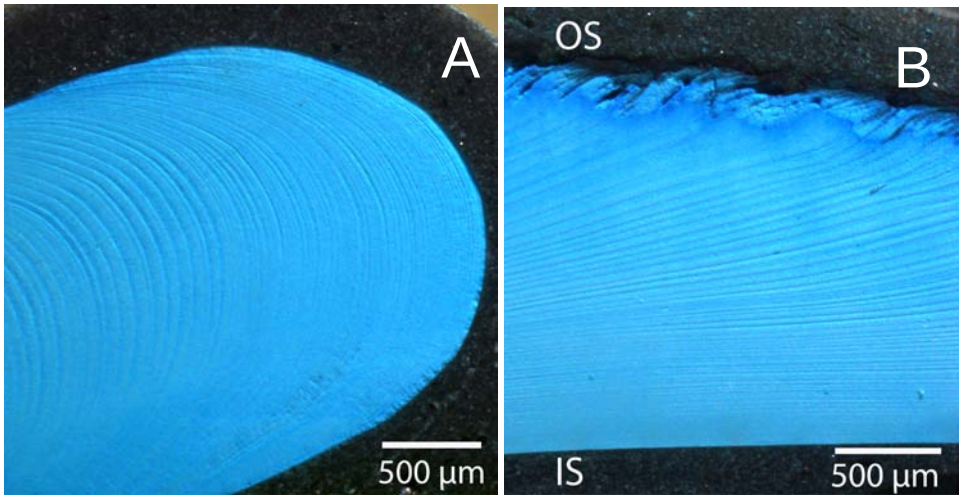

Fig. 2.6: Shells of *Arctica islandica*. Wide annual growth increments are separated by darker growth lines (A) in the umbo and (B) along the outer shell side (OS). IS = inner shell side.

3. PUBLICATIONS

The general concept of this study was developed by PD Dr. Doris Abele, and in some aspects by Prof. Dr. Ralf Dringen. The project was funded by the Deutsche Forschungsgemeinschaft (DFG grant numbers AB124/10-1 and DR262/10-1). The experimental and analytical work, as well as method development was carried out by myself with provision of laboratories and equipment by the Center for Biomolecular Interactions Bremen, University of Bremen (Prof. Dr. Ralf Dringen) and the section of Functional Ecology, Alfred Wegener Institute for Polar and Marine Research, Bremerhaven (laboratory of PD Dr. Doris Abele).
List of publications and declaration of my contribution towards them:

Publication I

Strahl J, Abele D (2010) Cell turnover in tissues of the long-lived ocean quahog *Arctica islandica* and the short-lived scallop *Aequipecten opercularis*. Marine Biology 157: 1283–1292

The scientific concept of this manuscript was developed by me and the second author. Sampling of *Arctica islandica*, experimental and analytical work as well as the evaluation of the data was performed by me. I wrote the manuscript, which was revised by the second author.

Publication II

Strahl J, Dringen R, Schmidt MM, Hardenberg S, Abele D (2011) Metabolic and physiological responses in tissues of the long-lived bivalve *Arctica islandica* to oxygen deficiency. Comparative Biochemistry and Physiology, Part A: Molecular & Integrative Physiology 158: 513–519

I developed the scientific concept of this study in discussions with D. Abele, R. Dringen and M. M. Schmidt. Sampling of the experimental animals, the main part of the laboratory work and the evaluation of the data was performed by me. The data set on glutathione was obtained by M. M. Schmidt and respiration measurements with isolated gills were carried out by S. Hardenberg as part of her diploma thesis. I wrote the manuscript, which was improved in cooperation with all co-authors.

Publication III

Strahl J, Brey T, Philipp EER, Thórarinsdóttir GG, Fischer N, Wessels W, Abele D (in revision). Metabolic rate depression: a key to longevity in the ocean quahog *Arctica islandica.* Journal of Experimental Biology

The scientific ideas for this paper were developed by me, in discussions with D. Abele and E. E. R. Philipp. Sampling of the experimental animals was conducted by me. Burrowing experiments in the field were performed by G. G. Thórarinsdóttir, who supplied the raw data for statistical

analysis. The laboratory work was carried out by me and in part by N. Fischer (PO_2 measurements in mantle cavity water) and W. Wessels (measurement of H_2O_2 formation in isolated gill tissue). The evaluation of the data was performed by me with assistance of T. Brey and N. Fischer. I wrote the manuscript, which was revised by D. Abele and the other co-authors.

PUBLICATION I

Cell turnover in tissues of the long-lived ocean quahog *Arctica islandica* and the short-lived scallop *Aequipecten opercularis*

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Abstract

Cell proliferation and apoptosis were investigated in tissues of two bivalve species, *Arctica islandica* from the German Bight (age of bivalves: 33-98 years) and Iceland (7-148 years) and *Aequipecten opercularis* from the English Channel (2-4 years). High proliferation rates (10% nuclei dividing) and apoptosis in tissues of *A. opercularis* were in line with high-energy throughput and reduced investment into antioxidant defence mechanisms in the scallop. In contrast, cell turnover was slow (<1% nuclei dividing) in *A. islandica* and similar in mantle, gill and adductor muscle between young and old individuals. In the heart, cell turnover rates decreased with age, which indicates less-efficient removal of damaged cells in ageing *A. islandica*. Cell turnover rates, mass specific respiration and antioxidant enzyme activities were similar in German Bight and Iceland ocean quahog. Variable maximum life expectancies in geographically separated *A. islandica* populations are determined by extrinsic factors rather than by fundamental physiological differences.

Keywords

Proliferation, apoptosis, antioxidant capacity, ageing, scallop, clam

Introduction

Bivalve maximum life span potentials (MLSP) vary between just a few and several hundreds of years, and a large variety of different life history models can be found among mollusks. Currently, bivalves are discussed as new models in ageing research that add to the understanding of the mechanistic basis of longevity with a focus on physiology and gene expression of long- and short-lived bivalve species (Abele et al. 2009; Bodnar 2009; Philipp and Abele 2010). The ocean quahog *Arctica islandica* with reported individual ages of >350 years in an Icelandic (IC) population (Schöne et al. 2005; Wanamaker et al. 2008) and the queen scallop *Aequipecten opercularis* with a MLSP of only 8–10 years, represent not only extremes in lifespans but also life styles. *Arctica islandica* is rather inactive, burrowing beneath the sediment surface but remaining in contact with the overlying sea water through short siphons for oxygen uptake and feeding. At regular intervals, the clams burrow deeper into the sediment exposing themselves to hypoxia for periods of 1−7 days and inducing metabolic rate depression (MRD) (Taylor 1976). When experiencing prolonged hypoxia of > 24 h, the ocean quahog reduces its metabolism to 10%, and after several days of experimental anoxia to as low as 1% of their aerobic rates (Oeschger and Storey 1993). *Arctica islandica* occurs on the continental shelves on both sides of the North Atlantic in Europe and North America (Nicol 1951; Thompson et al. 1980; Jones 1980; Murawski et al. 1982; Dahlgren et al. 2000), spanning an overall temperature range from 0°C to 16°C (Mann 1982). In contrast, *A. opercularis* is an actively swimming epibenthic scallop with a wide geographical distribution on the European continental shelf from Northern Norway to Gibraltar and within the Mediterranean (Waller 1991), tolerating water temperatures of 6-24°C (Ansell et al. 1991). The scallops swim through jet propulsion reaching speeds up to 30 cm s^{-1} (Moore and Trueman 1971).

Since the pioneer work of Pearl (1928) and Harman (1956), proposing the ´Free Radical - Rate of Living theory´, which predicts a negative correlation between mass specific respiration (MSR) and MLSP of a species, studies of ageing focus on the link between mitochondrial production of reactive oxygen species (ROS) and MSR. Applying this theory to the mollusks, a variety of physiological parameters such as growth, metabolism, antioxidant defence and accumulation of oxidative damage products have been studied in different tissues of several bivalves and squids in an age-dependent manner (Philipp et al. 2005a, b, 2006; Strahl et al. 2007; Abele et al. 2008; Zielinski and Pörtner 2000). The long-lived IC *A. islandica* has stable antioxidant capacities in mantle and gill over an age range of 30-200 years (Abele et al. 2008) and the fluorescent age pigment lipofuscin, an oxidative stress and ageing marker, accumulates in several tissues as they age (Strahl et al. 2007). The abundance of lipofuscin granules differs between highest densities in gills, less in mantle and hardly detectable accumulations in the adductor muscle of very old clams. The protein carbonyl concentration resulting from protein oxidation remains low in *A. islandica* up to 200 years compared to shorter-lived bivalves (Strahl et al. 2007). In contrast, mitochondrial volume density, as well as antioxidant protection decrease significantly in mantle tissue of ageing *A. opercularis,* within only a couple of years. Lipofuscin content in mantle tissue increases with age, and protein

carbonyls are twice as concentrated as in 200-year-old *A. islandica* (Philipp et al. 2006; Strahl et al. 2007). These results indicate that the energy-saving life style of *A. islandica* reduces oxygen radical damage accumulation and maintains high antioxidant capacities into extremely old age. In contrast, high-energy throughput and the epibenthic lifestyle of *A. opercularis* are correlated with a short life expectancy, due to an elevated risk of predation compared to burrowing bivalves, and a restricted investment in cellular protection and mitochondrial maintenance in mature specimens.

In the present work, we investigated tissue maintenance in terms of cell proliferation and apoptosis in mantle, gill, adductor muscle, and heart of both bivalve model species *A. islandica* and *A. opercularis*. Cell division and programmed cell death are essential processes in multicellular organisms, which eliminate and replace damaged, infected and senescent cells. Apoptosis is a highly regulated energy-consuming process in which intracellular compounds are degraded and disposed of in an orderly manner, without the induction of inflammation. Caspases, a family of proteases involved in the apoptotic program, have recently been described in bivalves (Sokolova et al. 2004; Sokolova 2009). Both, proliferation and apoptosis of fatally damaged cells may dilute and eliminate waste products such as lipofuscin and protein carbonyls, as well as damaged mitochondria in bivalve tissues, which thereby preserve function over an individual's lifetime. Young (7-13 years), middle aged (27-33 years) and old (93-148 years) IC *A. islandica* were studied to test for age-dependent capacity differences in proliferation and apoptosis in a long-lived organism. We included a second *A. islandica* population from the German Bight (GB), to test for possible intra-specific variability, and compared cell turnover rates as well as antioxidant enzyme activities and mass specific respiration rates between individuals from both populations. Ocean quahog from the GB have shorter life expectancy than the IC population and reported problems with new recruitment, which is possibly induced by environmental conditions and results in missing age cohorts <30 and >100 years (Begum et al. 2009).

Material and methods

Bivalve collection and maintenance

Arctica islandica were collected at Helgoland "Tiefe Rinne" in the GB (54°09.05'N, 07°52.06'E) at 40–45 m water depth, using a hydraulic dredge in May 2008. Surface water temperature on the sampling date was 12°C. In August 2008, *A. islandica* were collected northeast of Iceland (66°01.44'N, 14°50.91'W) at 8–15 m w ater depth and water surface temperatures of 9°C. Clams were transported in a cooled container to the Sandgerdi Marine Centre, University of Iceland, where they were kept for 2 weeks at constant temperature (9°C) and salinity (34 PSU) in 400-l tanks. *Aequipecten opercularis* were dredged in the English Channel (48°43.07'N, 03°59.02'E) in August 2008 at about 60-m water depth and 16°C surface temperature. *Arctica islandica* from the GB and IC and *A. opercularis* were transported in cooled containers to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven. Bivalves were acclimated for 2 months at 10°C

and 33 PSU in 60-l aquaria with re-circulating sea water containing 10 cm of pea gravel sediment. All bivalves were fed once a week with DT-live marine plankton (DT, USA, 3 ml bivalve^{-1} week^{-1}).

Measurement of proliferation rates

The measurement of proliferation rates in bivalve tissues was modified after Moore et al. (1994). For labelling with proliferation reagents, 5 GB *A. islandica,* 15 IC *A. islandica* of different age classes, and 5 *A. opercularis* were exposed individually in 3-l flasks filled with natural seawater (33 PSU) at constant water temperatures of 10°C. Three days prior to incubation, experimental bivalves were not fed any more to avoid eutrophication through faeces and microbial contamination of the incubation water. 5-Bromo-2-deoxyuridine (BrdU, Fluka, 30 mg l^{-1}) and 5-Fluoro-2-deoxyuridine (FdU, Fluka, 3 mg l^{-1}) were dissolved in the experimental water and taken up by the bivalves with the inhaled medium. 5-Fluoro-2-deoxyuridine reduces competition with endogenous thymidine and increases BrdU incorporation. Duration of exposure of *A. opercularis* was 24 h, and because of slower proliferation rates incubation time of IC and GB *A. islandica* was prolonged to 5 days. During the 5-days *A. islandica*-incubation, 1.5 l of the experimental medium was exchanged with an equal amount of freshly prepared BrdU and FdU seawater every 24 h. Control bivalves were held in seawater without proliferation reagents. Bivalves were dissected and blocks of gill, mantle, adductor muscle and heart tissue transferred to cryovials and snap frozen in liquid nitrogen for both proliferation and apoptosis measurements. The shells of all bivalves were cleaned and numbered for individual age determination. Tissue blocks were embedded on chucks using frozen section medium (Neg-50, Thermo Fisher Scientific, Waltham, USA) and cut with a cryostat HM 500 OM (MICROTOM GmbH, Walldorf, Germany) at -20°C in 1-µm-thick cryo-sections, which were placed on BioBond (BBInternational, West Chester, USA) coated slides. Cryosections were stained with anti-BrdU and diaminobenzidine (DAB) using an Amersham cell proliferation kit (GE Healthcare, Buckinghamshire, UK) with three changes to the standard protocol: (1) before rehydration, sections were fixed for 30 s in 100% acetone, washed 3 times in phosphate-buffered saline (PBS) and incubated 3 min in 3% H_2O_2, (2) 50 mM Tris-HCl buffer (50 mM Tris, 146 mM NaCl, 200 mM HCl) was used instead of PBS before staining with DAB, (3) eosin-phloxin solution was used to counterstain the chromogen for 30 s, this was followed by 6 dips in 80% ethanol, 10-min dehydration and embedding of the sections in Euparal (CHROMA-GESELLSCHAFT, 3C 239). Method controls were prepared from tissues of control bivalves and not labelled with BrdU, and tissues labelled with BrdU but using PBS to replace the primary antibody. A second cryosection of each sample was stained in haematoxylin and eosin-phloxin (H&E) to visualize all nuclei in the tissue section. Cryosections were analyzed with a Zeiss Axioscope light microscope equipped with a KS300 image analysis software to count both nuclei stained by H&E ($N_{H\&E}$) and dividing nuclei stained with the Amersham cell proliferation kit (N_P). Percent cell proliferation d^{-1} was calculated as:

$$\%\,proliferation\ 24\ h^{-1} = N_P / N_{H\&E} * 100 * d\ of\ incubation^{-1}$$

Measurement of caspase-3 and -7 activities

The measurement of apoptosis via caspase-3 and -7 activity was modified after Liu et al. (2004). Frozen samples of mantle, gill and adductor muscle of 5 GB *A. islandica,* 15 IC *A. islandica* of different age classes*,* and 5 *A. opercularis* were ground in liquid nitrogen and homogenized with a glass homogenizer (Nalgene, USA) in lysis buffer (100 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, and 1 µg ml^{-1} each leupeptin, pepstatin and aprotinin) 1:100 (w/v). Homogenates were centrifuged for 15 min at 15 000 *g* and 4°C, and apoptosis intensities in supernatants were determined as caspase-3 and -7 activities using a Caspase-Glo 3/7 Assay kit (Promega, Madison, USA). The assay provides a luminogenic caspase-3/7 substrate, diluted in a reagent optimized for caspase activity, luciferase activity and cell lysis. Equal volumes of reagents and supernatant were added to a white-walled 96-well plate and incubated at 25°C for 1 h. The proluminescent substrate was cleaved by caspases and extracted into the supernatant, where it formed a substrate for luciferase. The resulting luminescence signal is proportional to the amount of caspase activity present in the supernatant. Samples were analyzed using a Multilabel Reader LB 941 TriStar (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany) which gave luminescence readings as relative light units (RLU). Subsequently, protein concentration was determined in the supernatant according to Bradford (1976) and apoptosis intensities expressed as RLU mg^{-1} protein.

Measurement of superoxide dismutase activity

GB and IC *A. islandica* were dissected and gill and mantle tissues snap frozen in liquid nitrogen for enzyme activity measurements. Shells of all bivalves were collected for individual age determination. Superoxide-dismutase (SOD) activity was determined after Livingstone et al. (1992). 100–180 mg of frozen mantle and gill tissue were ground in liquid nitrogen and homogenized with a micropistill in Tris-HCl buffer (20 mM TRIS, 1 mM EDTA, 20 mM HCl, pH 7.6) 1:10 (w/v). Samples were centrifuged for 3 min at 18 000 *g* and 4°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. One unit SOD causes 50% inhibition under assay conditions. Mitochondrial and cytosolic SOD isoforms were not distinguished.

Measurement of catalase activity

Catalase (CAT) activity was determined after Aebi (1984). In phosphate buffer (50 mM KH_2PO_4, 50 mM Na_2HPO_4, pH 7.0) with 0.1% Triton x-100 at 1:30 (w/v), 20–50 mg of frozen mantle and gill tissue were ground in liquid nitrogen and homogenized with a micropistill. Samples were centrifuged for 15 min at 13 000 *g* and 4°C, and CAT activity was determined by recording the period required for H_2O_2 decomposition, resulting in a decrease in absorption from 0.45 to 0.4 at 240 nm (1 unit).

Calculation of respiration and metabolic rate

Respiration rates of GB and IC *A. islandica* were measured at 10°C in a multichannel intermittent flow system as described by Begum et al. (2009) and Heilmayer and Brey (2003). To eliminate the impact of specific dynamic action (SDA) on respiration, bivalves were maintained without food for three days (Bayne et al. 1976), and measured separately after one night in the respiration chamber. Oxygen concentration was monitored continuously with fluorescein-coated oxygen microoptodes (PSt1-L5-TF, PreSens, Neuweiler, Germany) connected to a MICROX TR3 array (PreSens, Neuweiler, Germany). After the measurement, clams were dissected and soft tissue dried at 68°C for at least 48 h to determine dry mass (DM). To obtain ash-free dry mass (AFDM = DM – ash) dried tissues of *A. islandica* were combusted for 24 h at 500°C. Oxygen consumption rates (VO_2, µmol O_2 h^{-1} ind^{-1}) and mass specific respiration rates (MSR, µmol O_2 h^{-1} ind^{-1} $AFDM^{-1}$) were calculated according to Begum et al. (2009). Shells of all bivalves were cleaned and numbered for individual age determination.

Age determination

Age determination for *A. islandica* was carried out as described by Schöne et al. (2004) and Strahl et al. (2007). Briefly, the right shell was embedded in epoxy resin and sectioned along the axis of maximum shell growth (height *H*) with a Buehler low-speed diamond saw. Cross-sections were ground and polished and annual shell growth bands were counted using a stereomicroscope at 10- to 80-fold magnification. In *A. opercularis,* individual age was inferred from shell height and a von Bertalanffy growth model (VBGM) established by Heilmayer et al. (2004) and based on height-at-age-data of the same scallop population from the English Channel. 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, k is the growth constant and t_0 is age at which size would be zero. To estimate age t from height of our experimental clams, we fitted the inverse VBGF:

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

Statistical analysis

Proliferation rates were transformed according to: $\arcsin\sqrt{\%\,proliferating\ cells\ \ 24\,h^{-1}\,100^{-1}}$. Statistical analyzes were performed with GraphPad Prism 5 Software (La Jolla, California, USA). Mass specific respiration rates, superoxide dismutase and catalase activity, and proliferation rates and apoptosis intensities of all three populations were tested for normality (Kolmogorov-Smirnov

test) and homogeneity of variances (Bartlett´s test). One-way ANOVA (Kruskal-Wallis test for non-Gaussian distributed data) with post-hoc test Tukey (Dunn´s) was used to test for differences in mass specific respiration and in superoxide dismutase and catalase activity in mantle and gill within the three populations. One-way ANOVA (Kruskal-Wallis test for no Gaussian distributed data) with post-hoc test Tukey (Dunn´s) was used to test for differences in proliferation rates and apoptosis intensities between tissues within one population and between three age groups within one tissue type. Two-way ANOVA and Bonferroni post-hoc test were used to test whether tissue type, species/population and age differentially affects proliferation rates or apoptosis intensities in different populations. Slopes of linear regressions were tested for deviation from zero with an *F* -test (proliferation rates and apoptosis intensities versus age for each tissue of all three populations).

Results

Proliferation rates and apoptotic intensity in tissues of A. islandica and A. opercularis

Proliferating cells were detected in mantle, gill, adductor muscle and heart of the two investigated species (Fig. 1). Proliferation rates and ratios between tissues were similar in GB and IC *A. islandica*, with slightly higher proliferation values in IC clams. GB *A. islandica* had significantly higher proliferation rates in gill tissue (0.8 ± 0.6% cell division in 24 h) compared to values < 0.1% cell division in 24 h in mantle and heart muscle (One-way ANOVA $P < 0.0001$, Tukey $P < 0.01$). Proliferation rates in GB *A. islandica* adductor muscle were below detection limit (= 0%) after 5 days of BrdU incubation. IC *A. islandica* mantle and gills had highest proliferation rates with around 1% of nuclei dividing in 24 h, followed by the heart muscle and significantly lower rates in adductor muscle with 0.22 ± 0.30% cell division in 24 h (Kruskal-Wallis $P < 0.001$, Dunn´s $P < 0.01$, Fig. 2, arcsin transformed data are shown). Proliferation rates were significantly higher in all tissues of the scallop *A. opercularis* compared to GB and IC *A. islandica* (Two-way ANOVA $P < 0.0001$; arcsin transformed data are given in Fig. 2). In gill tissue of *A. opercularis,* 12.3 ± 7.9% of nuclei were dividing within 24 h, whereas mantle, adductor muscle and heart were less actively proliferating without significant differences between scallop tissues. Apoptosis intensities within one tissue type were higher in *A. opercularis* compared to either GB or IC *A. islandica*, but only in adductor inter-specific differences were significant, with tenfold higher values in *A. opercularis* (One-way ANOVA $P < 0.0001$, Tukey $P < 0.001$, Fig.3). Mantle tissue had 3-times and gill 1.5-times higher apoptosis rates in *A. opercularis* compared to *A. islandica*. In the tissue comparison, highest apoptotic activity was observed in scallop gills, followed by adductor muscle and mantle. Again, differences were not statistically significant. Apoptosis intensity in gills of both GB and IC *A. islandica* ranged significantly higher than in mantle and adductor muscle, with eightfold lower values in adductor muscle and fivefold lower values in mantle (One-way ANOVA $P < 0.0001$, Tukey $P < 0.001$, Fig. 3).

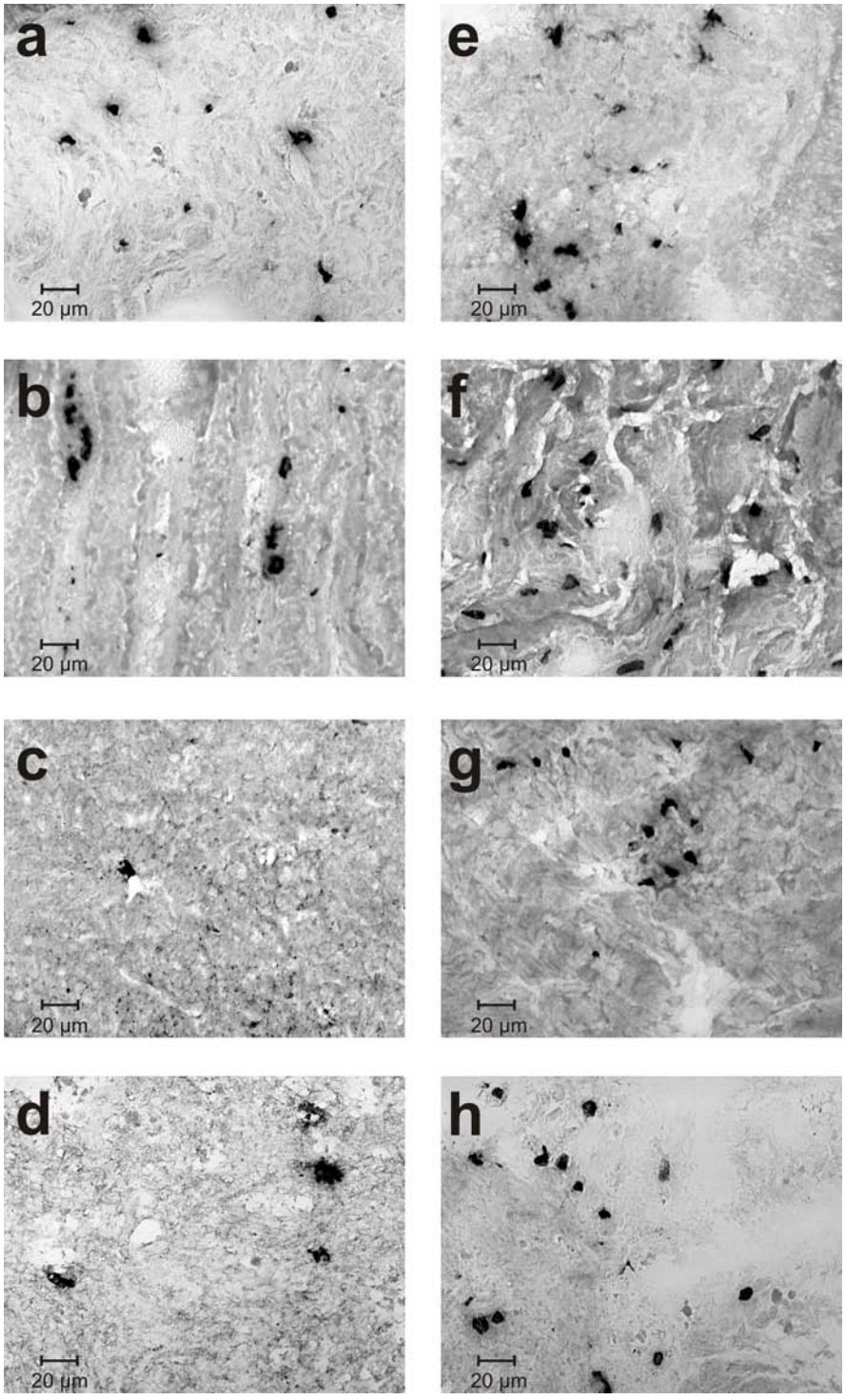

Fig. 1: Proliferating cell nuclei in cryo slices of mantle (a, e), gill (b, f), adductor muscle (c, g) and heart (d, h) of Icelandic *Arctica islandica* (left) after 5 days of BrdU incubation vs. *Aequipecten. opercularis* from the English Channel (right) after 24 h of BrdU incubation. BrdU-labeled nuclei appear black.

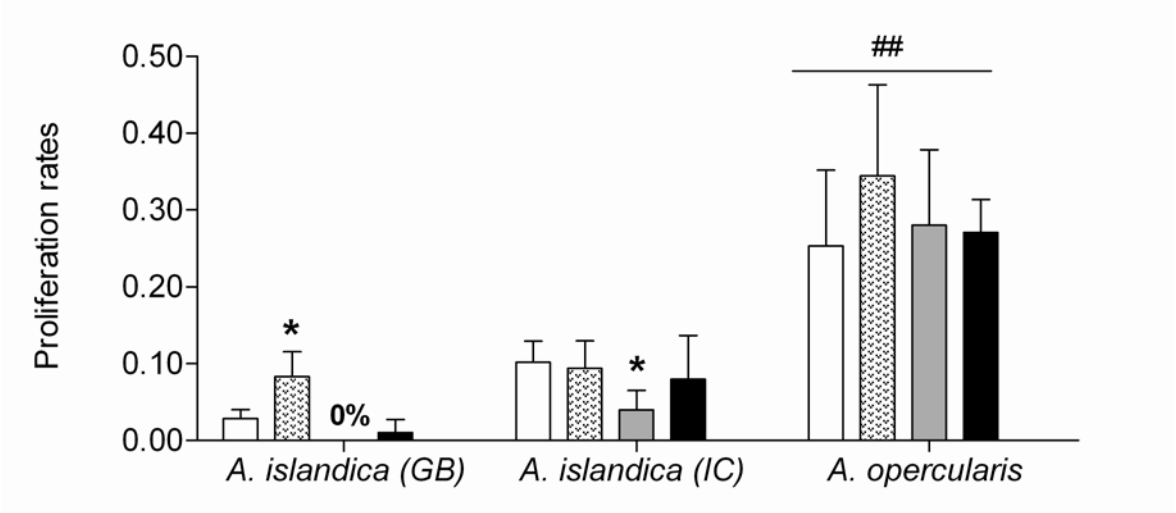

Fig. 2: Proliferation rates in arcsin √ (% proliferating cells $24h^{-1}$ 100^{-1}) in mantle (open bars), gill (dotted bars), adductor muscle (shaded bars) and heart (filled bars) of German Bight (GB) *Arctica islandica* (means ± SD, *n* = 5 per tissue type), Icelandic (IC) *A. islandica* (means ± SD, *n* = 15 per tissue type) and *Aequipecten opercularis* from the English Channel (means ± SD, *n* =5 per tissue type). ## significant differences in tissues of *A. opercularis* compared to GB and IC *A. islandica* (Two-way ANOVA P < 0.0001). * gill differs significantly from all other tissues in GB *A. islandica* (One-way ANOVA P < 0.0001; Tukey P < 0.01) and adductor muscle differs significantly from all other tissues in IC *A. islandica* (Kruskal-Wallis P < 0.001; Dunn´s P < 0.01). 0%: cell proliferation rate is zero in the adductor muscle of GB *A. islandica.*

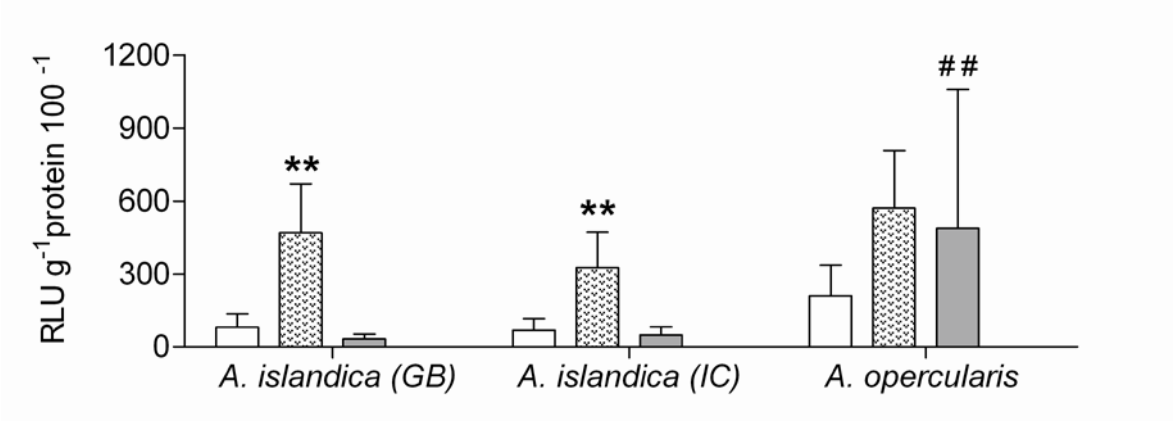

Fig. 3: Apoptosis intensities (RLU g^{-1}protein 100^{-1}) in mantle (open bars), gill (dotted bars) and adductor muscle (shaded bars) of German Bight (GB) *Arctica islandica* (means ± SD, *n* = 5 per tissue type), Icelandic (IC) *A. islandica* (means ± SD, *n* = 15 per tissue type) and *Aequipecten opercularis* from the English Channel (means ± SD, *n* = 5 per tissue type). ## significant differences in adductor muscle between *A. opercularis vs* both *A. islandica* populations (One-way ANOVA P < 0.0001, Tukey P < 0.001). ** significant differences in gill compared to other tissues of IC and GB *A. islandica* (One-way ANOVA P < 0.0001Tukey P < 0.001).

Proliferation rates and apoptosis intensities in tissues of IC A. islandica over an age range of 7 to 148 years

Proliferation rates decreased significantly with increasing age in the heart muscle of IC *A. islandica* (proliferation: One-way ANOVA P < 0.01; Tukey P < 0.05; Fig. 4, arcsin transformed data are

shown). Proliferation in the heart was 20 times lower in 118-148 years old clams than in young individuals of 7-10 years. Average rates in young clams were 1.93 ± 1.22% of proliferating heart cells in 24 h compared to 0.52 ± 0.49% in middle aged (27-32 years) and 0.10 ± 0.07% in old individuals (118-148 years) (linear regression $P < 0.05$, Fig. 6a, arcsin transformed data are shown).

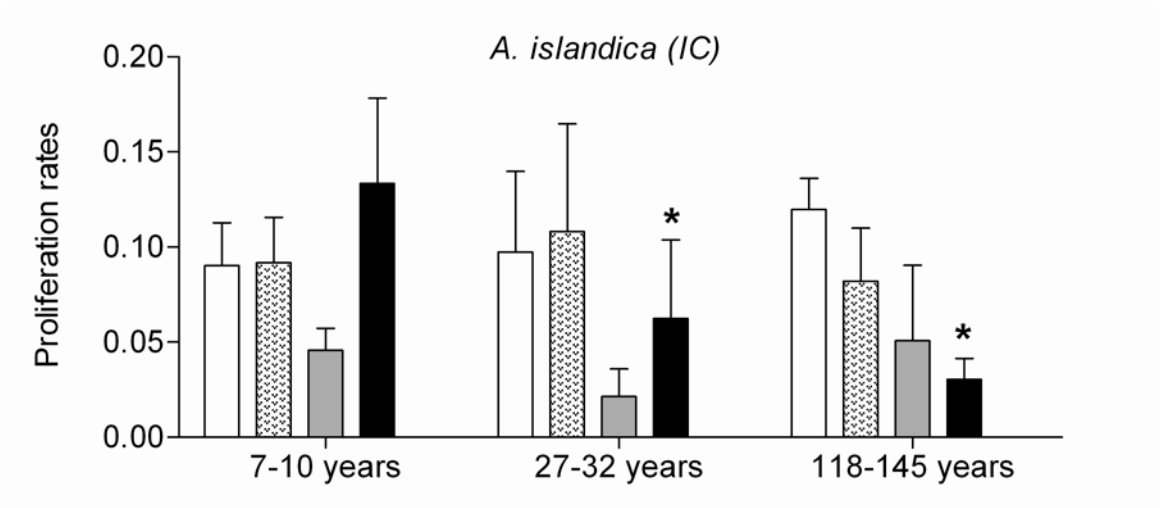

Fig. 4: Proliferation rates in arcsin(% proliferating cells 24h $^{-1}$ 100^{-1}) in mantle (open bars), gill (dotted bars), adductor muscle (shaded bars) and heart (filled bars) of young (7-10 years), middle aged (27-32 years) and old (118-148 years) Icelandic (IC) *Arctica islandica* (means ± SD, *n* = 5 per tissue type and age class). * significant differences between heart of middle aged and old compared to young individuals (One-way ANOVA $P < 0.01$; Tukey $P < 0.05$).

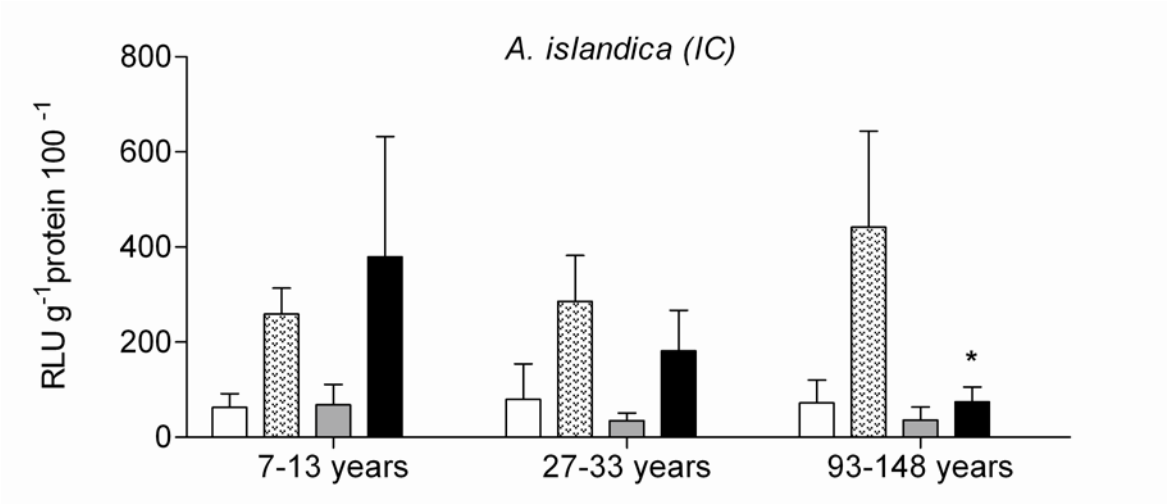

Fig. 5: Apoptosis intensities (RLU g^{-1}protein 100^{-1}) in mantle (open bars), gill (dotted bars), adductor muscle (shaded bars) and heart (filled bars) of young (7-10 years), middle aged (27-32 years) and old (118-148 years) Icelandic (IC) *Arctica islandica* (means ± SD, *n* =5 per tissue type and age class). * significant difference between heart of old compared to young individuals (One-way ANOVA $P < 0.05$; Tukey $P < 0.05$).

Also apoptosis intensities decreased significantly with age in the heart muscle of IC *A. islandica* (apoptosis: One-way ANOVA $P < 0.05$; Tukey $P < 0.05$; Fig. 5). Apoptotic intensity ranged highest

in heart tissue of young ocean quahog and was five-times lower in bivalves >100 years old (linear regression P < 0.05, Fig. 6b). In contrast to the heart values, proliferation rates and apoptotic activity in mantle, gill and adductor muscle of *A. islandica* were the same over an age range of 7−148 years (Fig. 4, 5).

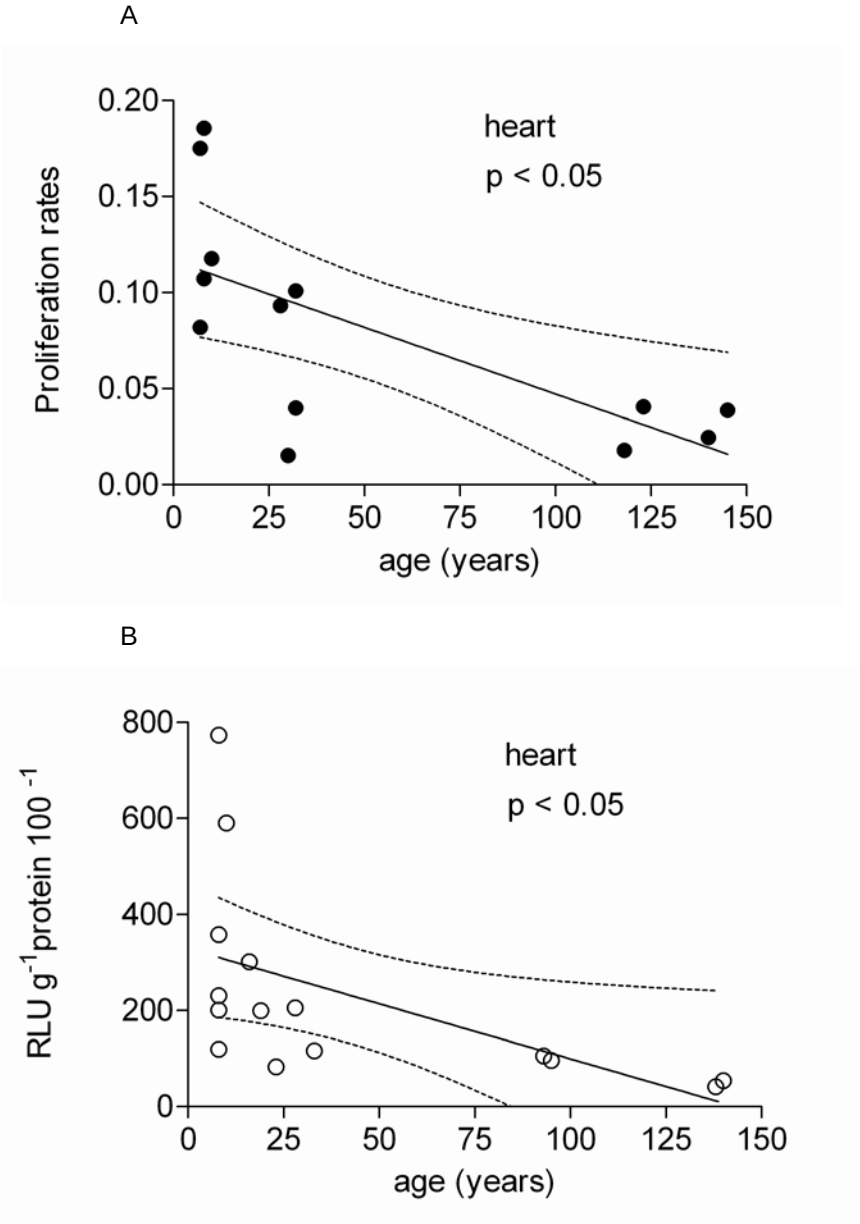

Fig. 6: a. Proliferation rates in arcsin √ (% proliferating cells $24h^{-1}$ 100^{-1}) (filled circles) ($y = -0.0007x + 0.117$; $r^2 = 0.462$; $n = 13$) and b. Apoptosis intensities in RLU g^{-1}protein 100^{-1} (open circles) ($y = -2.295x + 329.1$; $r^2 = 0.2907$; $n = 15$) in heart of Icelandic *Arctica islandica.*

Respiration rates and antioxidant enzyme activities in tissues of A. islandica and A. opercularis

Mass specific respiration rate (MSR) was significantly lower in GB and IC *A. islandica* compared to *A. opercularis* from the English Channel (Kruskal-Wallis P < 0.001, Dunn´s P < 0.005), which showed twice as high MSR. IC *A. islandica* had slightly higher MSR than GB clams, but differences

were not statistically significant (Table 1). In contrast both superoxide dismutase (SOD) and catalase (CAT) activity were significantly higher in mantle tissue of GB and IC *A. islandica* compared to *A. opercularis* (Kruskal-Wallis P < 0.0001, Dunn´s P < 0.05). SOD activity was twice as high in both *A. islandica* populations compared to *A. opercularis*. CAT activity was six-times higher in GB *A. islandica* and seven-times higher in IC *A. islandica* than in *A. opercularis.* No significant differences were detected in mantle or gill between the two *A. islandica* populations for either enzyme (Table 1).

Table 1: Investigated age range in years, mass-specific respiration rate (MSR) in µmol O_2 h^{-1} ind^{-1} $AFDM^{-1}$ and antioxidant enzyme activity of superoxide dismutase (SOD) and catalase (CAT) in U g^{-1}WM in tissues of German Bight (GB) and Iceland (IC) *Arctica islandica* and *Aequipecten opercularis* from the English Channel (MSR: *n* = 20 – 31 for each population/species, SOD/CAT: *n* = 6 – 11 for each population/species). ** Significant differences in MSR and CAT in mantle of *A. opercularis* compared to GB and IC *A. islandica* (Kruskal-Wallis P < 0.0001, Dunn´s P < 0.001). * Significant differences in SOD in mantle of *A. opercularis* compared to GB and IC *A. islandica* (Kruskal-Wallis P < 0.0001, Dunn´s P < 0.05). [a] Data for *A. opercularis* from Philipp et al. (2006), SOD and CAT activity recalculated in U g WM^{-1}. -[b] No data available.

	A. islandica (GB)	*A. islandica* (IC)	*A. opercularis*[a]
age	33-98	29-141	1-6
MSR	2.46 ± 0.40	4.05 ± 1.83	8.32 ± 3.91 **
SOD gill	728.62 ± 226.10	809.97 ± 406.53	-[b]
SOD mantle	790.04 ± 430.61	658.22 ± 364.18	379 ± 169 *
CAT gill	2944.35 ± 1016.08	3526.23 ± 740.30	-[b]
CAT mantle	2939.07 ± 633.21	2330.51 ± 452.43	384 ± 69 **

Discussion

We compared two bivalve model species, the long-lived and slow-growing ocean quahog *Arctica islandica* and the short-lived and fast-growing scallop *Aequipecten opercularis*. In the case of the IC ocean quahog, we could distinguish between age groups, whereas our entire sample of *A. opercularis* comprised 2−4 years old mature individuals still in the phase of highest growth. Von Bertalanffy growth models for *A. opercularis* from the English Channel demonstrate highest shell growth rates in conjunction with highest somatic production between 0 and 2 years of age, whereas from the 5th year on growth rates become asymptotic (Heilmayer et al. 2004). Rapid growth in young and mature *A. opercularis* of up to 4 years is in agreement with higher proliferation and cell turnover rates in tissues of the scallops compared to the ocean quahog. Rapid proliferation, especially in tissues such as the voluminous adductor muscle, cannot represent net-growth efficiency, because a muscle growth of 10% d^{-1} would exceed the slower shell growth within days. Indeed, high prolife-

ration is in part counterbalanced by a high intensity of programmed cell death. Next to early sexual maturation, starting at the age of <1 year (Román et al. 1996), short-lived *A. opercularis* are characterized by rapid cell turnover in somatic tissues, which explains the higher basic energy demand indicated by high mass specific respiration (MSR) compared to more slowly growing *A. islandica.* According to the ´Free-Radical-Rate-of-living´ theory of Pearl (1928) and Harman (1956) high oxygen turnover would suggest a high formation rate of reactive oxygen species (ROS) in the mitochondria. This could cause accelerated ageing and therefore be one reason why life span is limited to 10 years in *A. opercularis* (Ansell et al. 1991). However, mitochondrial production of ROS is hardly measurable in *A. opercularis* and ranged tenfold higher under the same assay conditions in *A. islandica* (Philipp et al. 2006, 2008; S. Hardenberg, D. Abele, unpublished data). Thus, scallops have higher mitochondrial respiration, but control ROS generation in tissues at extremely low levels, which presumably protects them from severe oxidative damage during frequent phases of exhaustive swimming. To conserve these low ROS levels over lifetime, *A. opercularis* eliminate damaged mitochondria from adductor muscle cells during ageing (Philipp et al. 2008). Permanent elimination of damaged mitochondria through intensive apoptosis is in keeping with absence of mitochondrial ageing in *A. opercularis*, and mitochondrial respiration and energetic coupling indeed do not change with age (Philipp et al. 2006).

Proliferation and apoptosis also eliminate oxidative waste products such as the fluorescent age pigment lipofuscin and protein carbonyls. Highest tissue cell turnover rates occur in gills of *A. islandica* and *A. opercularis*, possibly triggered by oxidative damage. The gill is one of the most active tissues and ROS production in this organ is assumed to be high. Accordingly, studies of lipofuscin accumulation in different tissues of the ocean quahog indicated better tissue protection and maintenance in adductor muscle and mantle compared to gills, where the age pigments were most highly concentrated (Strahl et al. 2007).

Arctica islandica is not only the longest lived bivalve known today, but moreover one of the slowest growing bivalves worldwide (Strahl et al. 2007). In addition to low MSR, slow gonad ripening and late onset of maturation at ages of 10−14 years in the ocean quahog (Thompson et al. 1980), slow cell proliferation and growth are additional criteria characterising long-lived species (Bauer 1992; Mangel 2003). These slow rates of cell division enable individual cells in ocean quahog tissues to grow to large diameters. Muscle fiber diameter increased fourfold in old (>100 years) compared to young clams (<10 years, Strahl et al. 2007). At the same time, low proliferation and cell turnover rates suggest that *A. islandica* cells should be endowed with an efficient system of waste removal through proteasomal and autophagic processes, and a well-established antioxidant defense system, to offset accumulation of cellular oxidative damage. Indeed, we found high and stable antioxidant protection in tissues of the ocean quahog between 30 and 200 years (Abele et al. 2008), whereas *A. opercularis* has a low investment in cellular protection and maintenance at advanced age (Philipp et al. 2006). Similarly, in the oyster *Crassostrea virginica* with a maximal live span (MLSP) of only 6 years, not only antioxidant enzyme activity was lower but, the density of apoptotic and

mitotic cells was also diminished in gill, adductor muscle and heart of mature compared to young oysters (Ivanina et al. 2008; Sunila and LaBanca 2003). It would be interesting to see whether in short-lived scallops the reported shift in energy allocation from somatic maintenance towards reproduction at the end of life (see also Heilmayer et al. 2004; Abele et al. 2009) leads to reductions of cell proliferation in somatic tissues, and whether or not this is accompanied by a decline of apoptotic activity. On the other hand, low metabolic activity and sustained protection from oxidative damage over age, in combination with slow but continued cell turnover, presumably slow senescent decline in *A. islandica*. Long-lived mollusks are known to budget lifetime energy investments equally between somatic maintenance and persistent but slow rates of gametogenesis (Arntz et al. 1994) and the lifelong continued damage repair and cell turnover explains negligible senescence in long-lived species (Kirkwood 2005; Ebert 2008).

The present study indicates negligible evidence of senescence in the mantle, gill and adductor muscle, whereas proliferation and apoptosis in the heart of *A. islandica* decreased dramatically with age. Since studies in the 1920s reported difficulties in detecting dividing cells or mitosis in the human heart (Karsner et al. 1925), the adult heart was regarded as postmitotic tissue. In contrast, we found proliferating heart cells in mature scallops and ocean quahog of all age classes, which is in line with more recent studies on vertebrates and suggests that heart cells of vertebrates and invertebrates can proliferate until old age and thus regenerate heart functions to some extent (Anversa et al. 1991; Kajstura et al. 1998; Poss et al. 2002). However, decreasing cell turnover rates over a lifetime of 140 years in the ocean quahog heart were indeed the only symptoms of old age indicating less-active cell renewal through proliferation and less-efficient removal of damaged cells. This in turn may indicate higher damage levels and possibly ongoing loss of function in the heart of ageing *A. islandica*. Impaired cardiac function has been suggested to limit life span in mammals (Leri et al. 2003) and perhaps ´heart failure´ could finally cause natural death in old ocean quahog.

Our second question was whether geographically distant populations of *A. islandica* from the GB and from IC, that differ by at least 100 years in the recorded maximum age (Begum et al. 2009), are also physiologically distinct. Our measurements documented no significant differences between the two populations for proliferation, apoptosis, respiration rates and antioxidant protection. Thus, a difference of 5°C in bottom habitat temperature between the southern North Sea and the North Iceland fishing grounds was not accompanied by differences in ocean quahog physiology that would explain the shorter life span of *A. islandica* in warmer North Sea waters. This contrasts with other studies which, either by comparing latitudinal populations of the same species (Ziuganov et al. 2000) or bivalves from similar habitats in temperate and polar habitats (Philipp et al. 2005a, b, 2006), document longer life span and slower change of physiological parameters with age in the cold-adapted populations/species. Similar physiological capacities are, however, consistent with observations of a low level of genetic differentiation between climatically distinct *A. islandica* populations throughout the NE Atlantic (Dahlgren et al. 2000; Held et al. in prep).

Age-dependent measurements of physiological parameters in GB *A. islandica* would have allowed a comparison of trends with age with the IC population, but were impossible, because individuals <30 years old are missing, and older individuals >100 years are rarely encountered in the GB. Instead, our sample was limited to 32-98 years old clams with an average shell height of 74 ± 5 mm. The narrow age range was earlier reported by Witbaard and Bergman (2003) who studied populations in the south-eastern North Sea between 1990 and 2000 and found them dominated by adults of 50−85 mm shell height, whereas young clams of smaller size were extremely rare. The relative absence of young individuals either indicates problems with larval settlement and recruitment, or high mortality rates of very young *A. islandica* during the past 35 years in the southern North Sea (Witbaard and Bergman 2003). An important impact comes from beam trawl fishery which caused a general decline in the number of benthic bivalves in the past 100 years in the southern North Sea (Rumohr and Kujawski 2000) and mainly affects young *A. islandica* (Witbaard and Klein 1994). Moreover, environmental change in the GB area, including strong eutrophication in the 1960s to 1990s (Rijinsdorp and van Leeuwen 1996) and slow but steadily increasing water temperatures around Helgoland (Wiltshire and Manly 2004), could cause thinning of the *A. islandica* population and the reduction of individual life expectancy. Indeed, conditions around IC are more favorable in many respects including food availability and absence of severe fishery impacts (Witbaard et al. 1999; Kaiser et al. 2006), which explains the higher densities of IC *A. islandica* (Ragnarsson and Thórarinsdottier 2002; Witbaard and Bergman 2003).

Our study suggests that maximum life expectancy in long-lived ocean quahog populations is mainly constrained by environmental impacts in different areas and does not reflect differences in the basic physiology and cellular renewal capacities between North Sea and IC *A. islandica*. Low metabolic rates and slow but stable cell turnover in tissues of IC *A. islandica* over 140 years, combined with high antioxidant protection with age, characterized the long-lived and slow-growing ocean quahog in both regions. In contrast the short-lived and fast-growing scallop *A. opercularis* is characterized by high-energy throughput, reduced investment in cellular protection and high proliferation and apoptosis intensities. Low rates of cell proliferation and apoptosis in the heart of IC *A. islandica* were the only symptoms of old age, which may indicate higher damage levels and ongoing loss of function. Future studies of ageing in mollusks should look into cellular viability and oxidative stress parameters in the heart.

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PUBLICATION II

Metabolic and physiological responses in tissues of the long-lived bivalve *Arctica islandica* to oxygen deficiency

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Abstract

In *Arctica islandica*, a long lifespan is associated with low metabolic activity, and with a pronounced tolerance to low environmental oxygen. In order to study metabolic and physiological responses to low oxygen conditions vs. no oxygen in mantle, gill, adductor muscle and hemocytes of the ocean quahog, specimens from the German Bight were maintained for 3.5 days under normoxia (21 kPa = controls), hypoxia (2 kPa) or anoxia (0 kPa). Tissue levels of anaerobic metabolites octopine, lactate and succinate as well as specific activities of octopine dehydrogenase (ODH) and lactate dehydrogenase (LDH) were unaffected by hypoxic incubation, suggesting that the metabolism of *A. islandica* remains fully aerobic down to environmental oxygen levels of 2 kPa. PO_2-dependent respiration rates of isolated gills indicated the onset of metabolic rate depression (MRD) below 5 kPa in *A. islandica,* while anaerobiosis was switched on in bivalve tissues only at anoxia. Tissue-specific levels of glutathione (GSH), a scavenger of reactive oxygen species (ROS), indicate no anticipatory antioxidant response takes place under experimental hypoxia and anoxia exposure. Highest specific ODH activity and a mean ODH/LDH ratio of 95 in the adductor muscle contrasted with maximal specific LDH activity and a mean ODH/LDH ratio of 0.3 in hemocytes. These differences in anaerobic enzyme activity patterns indicate that LDH and ODH play specific roles in different tissues of *A. islandica* which are likely to economize metabolism during anoxia and reoxygenation.

Keywords

Arctica islandica, hemocytes, hypoxia, anoxia, metabolic rate depression, anaerobic metabolism, glutathione

Introduction

The long-lived ocean quahog, *Arctica islandica,* with reported individual ages of more than 350 years (Schöne et al. 2005; Wanamaker et al. 2008) is among a few species in the Baltic and the North Sea which survive frequent and prolonged exposures to hypoxia and anoxia (Oeschger and Storey 1993; Diaz and Rosenberg 1995). The bivalves colonize pea gravel and muddy bottom sediments and constitute a major biomass component among the hypoxia tolerant survivor species in eutrophicated areas (Rosenberg et al. 1992). A low standard metabolic rate, slow but stable cell turnover rates, and high antioxidant protection levels that are maintained into old ages up to 200 years support the extreme longevity of this species (Abele et al. 2008; Strahl and Abele 2010).

The capacity of *A. islandica* to survive oxygen limited conditions is presumably rooted in its energetically economized lifestyle, combining extremely slow growth with low oxygen consumption rates (Begum et al. 2009). *Arctica islandica* reduces metabolic expenditure, when being confronted to environmental hypoxia or anoxia. Thus, caloric heat production in Baltic Sea specimens fell to 1% of fully aerobic rates after 60 days of experimental anoxia exposure (Oeschger 1990). During prolonged burrowing and shell closure of > 24 h bivalves from the Irish Sea lowered heart beat to 10 % of normoxic beat frequency (Taylor 1976). Spontaneous burrowing and shell closure cause hypoxia in mantle cavity water and are frequently observed in undisturbed *A. islandica* maintained in normoxic seawater (Taylor 1976; Abele et al. 2010).

In addition to energy saving behavior, *A. islandica* is, like other hypoxia tolerant ectotherms, endowed with a well established anaerobic energy producing system (Taylor 1976; Livingstone et al. 1983; Oeschger 1990; Oeschger and Storey 1993). Bivalves from the Baltic Sea switch to anaerobiosis during the first 48 h of experimental exposure to anoxia and can survive extended periods of time exclusively on glycolytic and mitochondrial anaerobic ATP production (Oeschger 1990). Pyruvate dehydrogenases such as octopine dehydrogenase (ODH), alanopine, strombine and lactate dehydrogenases (LDH) guarantee the continuous flux of glycolysis and, consequently, a constant supply of ATP by maintaining a low cytosolic redox-status ($NADH/NAD^+$ ratio) during anaerobiosis (Gäde and Grieshaber 1986). Activities of all four pyruvate dehydrogenases were detected in muscle tissue of *A. islandica* (Livingstone et al. 1983). LDH activity is reported to be low in mollusks compared to other invertebrates such as crustacea or insects, whereas activities of enzymes essential for octopine and succinate production are high (Livingstone et al. 1983; Grieshaber et al. 1994). Maximal lactate concentrations in the hemolymph of five-day burrowed *A. islandica* from the Irish Sea amounted to only 5 µg ml^{-1} (Taylor 1976) compared to 5200 µg ml^{-1} in crayfish hemolymph after 16 h of anoxic exposure (Grieshaber et al. 1994). No data exist on octopine or lactate formation in different tissues of the ocean quahog, but succinate, propionate and acetate accumulate in adductor and foot muscle of Baltic Sea *A. islandica* after two days of experimental anoxia (Oeschger 1990).

A yet unresolved question is whether there is a difference between environmental hypoxia and anoxia for the metabolic and physiological response of *A. islandica.* Mostly anoxia is simply

regarded as an ‘extreme case of environmental hypoxia’ but, indeed, this may not be so for extremely hypoxia tolerant and yet basically aerobic species such as *A. islandica*. So, is there a low tissue PO_2 threshold in hypoxia tolerant mud clams down to which aerobic energy metabolism can be maintained by economizing energy expenditures, and below which the animals need to switch to anaerobiosis? Taylor (1976) found that the mantle cavity water of five-day burrowed *A. islandica* still contained low quantities of oxygen and had a PO_2 of 1.3 kPa. As the accumulation of anaerobic metabolites was not determined in his study, it is not clear whether the mitochondria were working at very slow aerobic rates or had already started anaerobic energy production.

Maintaining the mitochondria in a slow performing and aerobic state and, thereby, avoiding the complete reduction of electron transport chain intermediates, could support longevity in *A. islandica*, as these reduced mitochondrial complexes are held responsible for the enhanced formation of ROS during reoxygenation. Cellular ROS are mainly generated during mitochondrial respiratory activity (Turrens 2003; Balaban et al. 2005) and the conversion of oxygen to H_2O_2 in invertebrate mitochondria is usually less than 1% of the oxygen consumed under phosphorylating state III conditions (Abele et al. 2007). The absolute amount of cellular ROS production in marine invertebrates depends on tissue oxygenation (Buchner et al. 2001) as well as on the number and the site specific redox potential of mitochondrial chain units (Balaban et al. 2005). Thus, our *a priori* assumption is that ROS production should be low in tissues of *A. islandica* under normoxia (see also Buttemer et al. 2010, Table 1) and even lower in a metabolically down-regulated state, in which electron transport and oxidative phosphorylation are slow but functioning. Nevertheless, the antioxidant defence system, including the synthesis of ROS-scavenging glutathione (GSH), may be up-regulated during MRD in *A. islandica* to prevent oxidative stress during reoxygenation. This has been detected in several hypoxia- and anoxia-tolerant invertebrates undergoing estivation and freezing (Hermes-Lima and Zenteno-Sávin 2002).

The aim of the present study is the investigation of tissue-specific responses of *A. islandica* to severe hypoxia and anoxia. In order to compare the activity of LDH and ODH in tissues and hemocytes, to measure the contents of anaerobic metabolites and of GSH as well as to determine the cellular thiol redox state, bivalves from the German Bight were incubated for 3.5 days under normoxic, hypoxic or anoxic conditions. Oxygen consumption was measured in isolated gills of *A. islandica* pre-incubated at normoxic or hypoxic PO_2, in order to confirm the effect of diminishing oxygen availability on metabolic rate in the organ most directly exposed to environmental oxygenation.

Materials and Methods

Bivalve collection and maintenance

Arctica islandica were collected in May 2008 at Helgoland “Tiefe Rinne” in the German Bight (54°09.05’N, 07°52.06’E) at 40-45 m water depth, using a trawl net. Clams were transported in cooled containers to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven.

Bivalves were held at 10°C and 33 PSU in 60-l aquaria with recirculating seawater containing 10 cm of gravel sediment and were fed once a week with a mixture of *Nannochloropsis occulato*, *Phaeodactylum tricornutum* and *Chlorella sp*. (DT´s plankton farm, USA, 3 ml bivalve^{-1} week^{-1}).

Materials

Unsterile 96-well plates were purchased from Sarstedt (Karlsruhe, Germany). Glutathionedisulfide (GSSG) and glutamate phosphate were obtained from Fluka (Buchs, Switzerland). Octopine, octopine dehydrogenase, 5-5´-dithio-bis-(2-nitrobenzoic acid), tri-sodium citrate and ethylenediamine tetraacetic acid were purchased from Sigma (Steinheim, Germany). NADPH, NADH (disodium salt), NAD^+, L-arginine-hydrochloride, sulfosalicylic acid and perchloric acid (60 %) were obtained from Applichem (Darmstadt, Germany). Alpha-D-glucose was obtained from Serva (Heidelberg, Germany) and glutamate pyruvate transaminase and GSSG reductase from Roche (Mannheim, Germany). All other chemicals, in the highest purity available, were purchased from Merck (Darmstadt, Germany) and Fluka.

Incubation experiments and sample preparation

Arctica islandica were exposed individually in 3-l flasks filled with natural seawater at constant water temperatures of 10°C and salinity of 33 PSU. Three days before the start of the experiment, bivalves were not fed any more to avoid eutrophication through faeces and microbial contamination of the experimental water. *Arctica islandica* were incubated under normoxic conditions (21 kPa) or under hypoxic conditions (2 kPa) by using a gas-mixture of nitrogen and oxygen (Air liquid, Germany) or under complete anoxia (0 kPa). Siphon-status of each individual was visually checked and recorded 3 times per day during incubations. After 3.5 days of incubation, shell closure of each bivalve was prevented by inserting a metal bar, 3 mm thick and 3 cm long. Then hemolymph was taken from the adductor muscle of each individual with a sterile needle and a 10 ml-syringe to determine enzyme activities and concentrations of anaerobic metabolites. Subsequently bivalves were dissected. One fresh sample-set of mantle, gill, adductor muscle and hemocytes was used to determine enzyme activities and the contents of total glutathione (GSx = GSH + 2x GSSG) and of GSSG. A second sample-set of each tissue type and of hemolymph without hemocytes was snap frozen in liquid nitrogen for laboratory analysis of anaerobic metabolites. The third group of bivalves was incubated under complete anoxia in order to confirm that our hypoxic incubation period covered the time window in which anaerobic metabolism can be induced in German Bight *A. islandica* and to test for octopine, lactate and succinate accumulation. Samples of under normoxia- and anoxia-incubated bivalves stored at -80 °C were further analyzed for the specific GSx content.

Determination of LDH and ODH

Freshly dissected mantle, gill and adductor muscle samples of bivalves incubated under normoxia or hypoxia were homogenized on ice with an ultra-turrax (IKA-Werke, Germany) after adding a 6-fold volume (w/v) of homogenizing-buffer (20 mM Tris-HCL, 1 mM EDTA, 1 mM DTT, pH 7.5). The homogenate was centrifuged for 15 min at 12,000 g and 4°C. Fresh hemolymph was

transferred into a 10 ml-tube and centrifuged for 10 min at 500 g and 4°C. The pellet containing the hemocytes was dissolved in 20 mM Kpi buffer and incubated on ice for 10 min. The sample was centrifuged for 1 min at 12,000 g and 4°C and LDH and ODH activities were determined in supernatants of tissues and hemocytes according to Livingstone et al. (1990). The absorbance decrease of NADH at 340 nm was recorded over 10 min in 15 sec intervals in a microplate reader (Sunrise, Tecan, Germany). Reactions were run in triplicate in the absence and presence of arginine to calculate the ODH activity by subtracting the LDH activity from the total enzyme activity.

Determination of octopine, lactate and succinate

Frozen mantle, gill and adductor muscle samples of bivalves incubated under normoxia, hypoxia or anoxia were homogenized on ice with an ultra-turrax and ultrasound (Bandelin, Germany) after adding a 6-fold volume (w/v) of 0.5 M perchloric acid. The homogenate was centrifuged for 15 min at 12 000 g and 4°C and the supernatant of each sample was neutralized with 2 M KOH and centrifuged for 5 min at 12 000 g and 4°C. Octopine and lactate contents of all tissue samples and of defrosted hemolymph, mantle cavity water and incubation water samples were determined after Luisi et al. (1975) and Schmidt and Dringen (2009), respectively. The reaction mixture for octopine quantification contained 0.25 U of ODH per well (180 µl), that for lactate quantification 7.15 U LDH per well. After incubation for 90 min at room temperature (RT) the absorbance of NADH was measured at 340 nm in the microplate reader. In the adductor muscle samples additionally the succinate content was determined after Michal et al. (1976) using the succinic acid Assay kit (Cat. No. 10 176 281 035, Boehringer Mannheim/R-Biopharm, Germany). The absorbance of NADH was recorded in UV-DU 800 spectrophotometer (Beckmann, Germany) at 340 nm and 37 °C. The incubation time was prolonged to 30 min to allow complete reaction of all the succinate in the sample. Contents of metabolites are shown per mg wetmass (WM).

Determination of glutathione

Freshly dissected mantle, gill and adductor muscle samples of specimens incubated under normoxia and hypoxia, as well as frozen samples of bivalves incubated under anoxia, were homogenized on ice using an ultra-turrax and ultrasound in a 6-fold volume (w/v) of 1% sulfosalicylic acid (SSA). Samples were centrifuged for 1 min at 12,000 g and 4°C. One milliliter of fresh hemolymph of each of the individuals incubated under normoxia or hypoxia was centrifuged for 10 min at 500 g and 4°C, respectively. The pellet containing the hemocytes was diluted in 100 µl of 1% SSA, incubated on ice for 10 min and centrifuged for 1 min at 12,000 g and 4°C. The contents of GSx and GSSG in the supernatants of tissues and hemocyte lysates were determined as described previously (Dringen and Hamprecht 1996) in microtiter plates according to the colorimetric method originally described by Tietze (1969). The detection limit of this assay is about 0.2 nmol GSx per 500 µl lysate. Specific GSx and GSSG contents were obtained by normalizing the GSX and GSSG contents of the tissue supernatants to the protein content of the respective acid precipitated pellets of tissues and hemocyte samples.

Determination of protein content

The pellets of tissues and hemocyte samples used for the determination of GSx were air-dried for 30 min, homogenized with ultrasound in 0.5 M NaOH and incubated for 2 h at RT. Protein contents of these homogenates as well as of the homogenates used for ODH and LDH enzyme activity assays were determined according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Measurement of gill respiration rates at three different PO_2

Bivalves were kept without food for three days before the experiment to eliminate the impact of specific dynamic action on gill respiration (Bayne et al. 1976). Gills of *A. islandica* were freshly dissected and transferred in cooled respiration-buffer (450 mM NaCl, 10 mM KCl, 20 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 0.5 mM DTT, 0.055 mM glucose, pH 7.4). Gill filaments were cut into 3 pieces of 15-25 mg WM and incubated for 2 h in respiration-buffer at 8 °C and with PO_2 adjusted to 2 kPa, 5 kPa or 21 kPa, respectively. Low PO_2 of 2 kPa and 5 kPa were adjusted with gas-mixtures of nitrogen and oxygen. Following incubation, each piece of gill was transferred into a refrigerated respiration chamber filled with 1.1 ml respiration-buffer at 8 °C and adequate PO_2. Each chamber was equipped with a net fixed on a plastic ring to protect the gill from the magnet stirrer at the bottom of the chamber and the chambers were closed hermetically with a stopper. The oxygen consumption rates of gill pieces were measured for 30 min at 2 kPa, 5 kPa or 21 kPa using single channel Microx TX-3 oxygen meters equipped with oxygen needle-optodes (PSt1-L5-TF, Precision Sensing GmbH, Germany) which were introduced into the chamber through a borehole in the stopper. Prior to measurements, optodes were calibrated to 100% air saturation with aerated seawater and to 0% using a saturated ascorbate solution. After respiration measurements the WM of each gill piece was determined and respiration rates were calculated as nmol O_2 min^{-1} ml^{-1} mg^{-1}WM.

Statistical analyses

Statistical analyses were performed with GraphPad Prism 5 Software (La Jolla, California,USA). All data sets were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Bartlett's test) before testing for tissue and PO_2 specific differences in enzyme activities, accumulation of anaerobic metabolites, GSx content or gill respiration.

Results

Siphon behavior, enzyme activities and anaerobic metabolites in tissues and hemocytes or hemolymph of A. islandica

All animals survived 3.5 days of experimental exposure to hypoxia and anoxia. We observed their siphons to be permanently open during hypoxic and anoxic incubation, whereas bivalves exposed to fully normoxic oxygenation alternately opened and closed siphons.

Neither LDH nor ODH activities in mantle, gill, adductor muscle and hemocyte cells differed significantly under hypoxic compared to normoxic incubation conditions (Fig. 1A, B). However, tissue specific differences in the ratio of both enzymes were observed. The adductor muscle had significantly higher ODH activity than all other tissues (Fig. 1B) and the highest ODH/LDH ratio (normox/hypox = 95.1 ± 145.4), whereas enzyme ratios in mantle (normox/hypox = 1.1 ± 0.9), gill (normox/hypox = 0.3 ± 0.3) and hemocytes (normox/hypox = 0.3 ± 0.3) were significantly lower under normoxic and hypoxic conditions (Kruskal-Wallis $P < 0.0001$; Dunns $P < 0.05$).

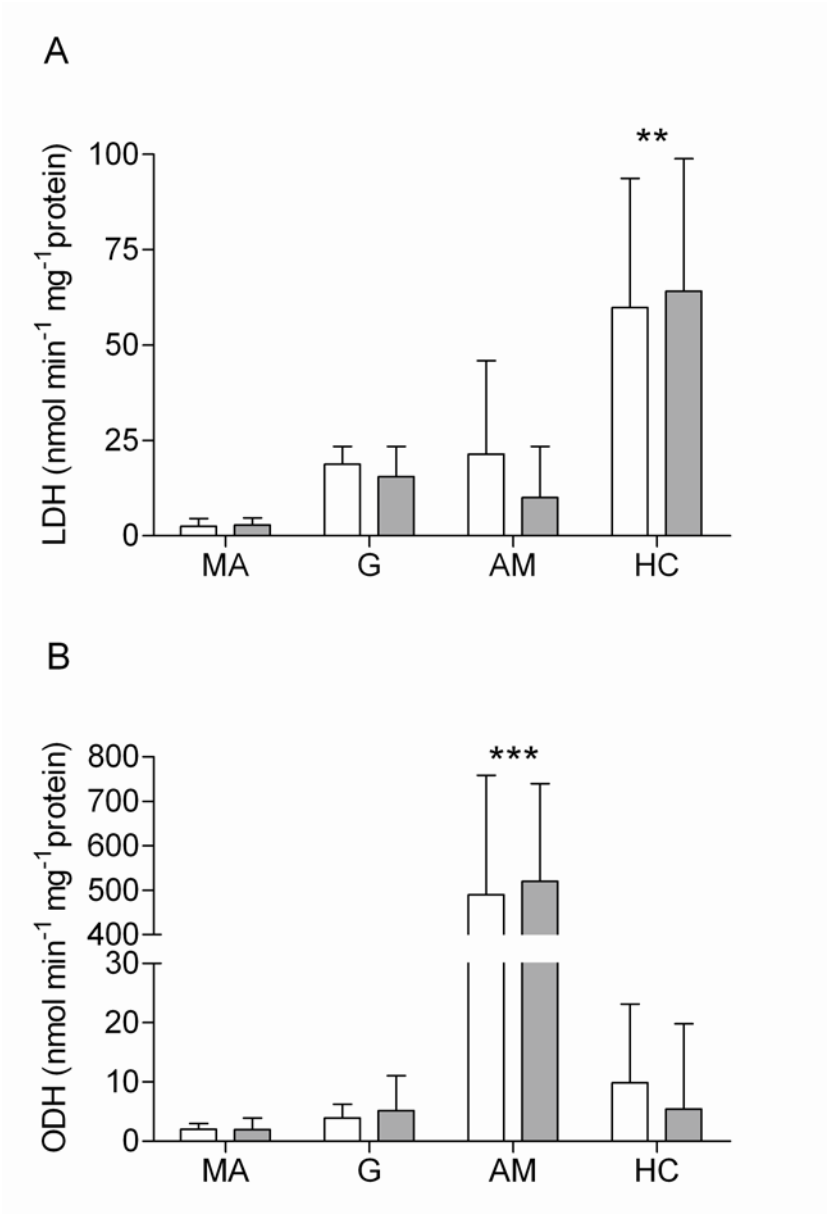

Fig. 1: Activities of (A) lactate dehydrogenase (LDH) and (B) octopine dehydrogenase (ODH) in mantle (MA), gill (G), adductor muscle (AM) and hemocytes (HC) of *Arctica islandica* incubated under normoxic conditions with a PO_2 of 21 kPa (open bars) or under hypoxic conditions with 2 kPa (filled bars), mean ± SD, *n* = 7-10 per tissue type and treatment. ** LDH activity in hemocytes differs significantly from that of all tissues under normoxic and hypoxic conditions (Two-way ANOVA $P < 0.0001$; Bonferroni $P < 0.01$). *** ODH activity in adductor muscle differs significantly from that of hemocytes and of all other tissues under normoxic and hypoxic conditions (Two-way ANOVA $P < 0.0001$; Bonferroni $P < 0.001$).

Hemocytes had significantly higher LDH activity than the other tissues (Fig. 1 A), and both, LDH and ODH activities were lowest in the mantle (Fig. 1 A, B).

Among the tissues investigated, octopine was only detectable in the adductor muscle and amounted to 10.5 ± 5.1 pmol mg^{-1} WM under normoxic conditions (*n*=10), 9.4 ± 5.3 pmol mg^{-1} WM under hypoxic conditions (*n*=7) and 9.1 ± 6.5 pmol mg^{-1} WM under anoxic conditions (*n*=10), indicating that 3.5 days of hypoxic or anoxic exposure do not accelerate octopine formation in *A. islandica*. Succinate contents remained unaltered in adductor muscle between clams exposed to normoxia or hypoxia, but were significantly elevated after the same period of anoxia (Fig. 2). The lactate content remained below the detection limit in all tissues including hemolymph and in the incubation water under normoxic, hypoxic and anoxic conditions.

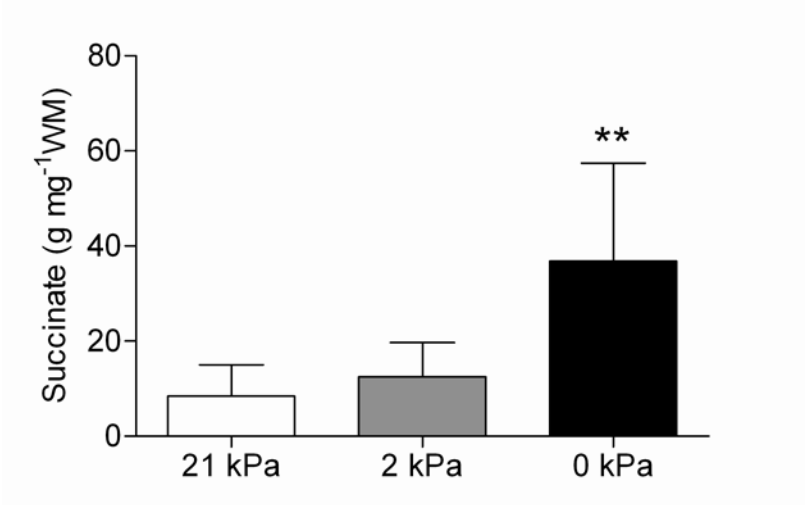

Fig. 2: Succinate content in the adductor muscle of *Arctica islandica* incubated under normoxic conditions with a PO_2 of 21 kPa, hypoxic conditions with 2 kPa and anoxic conditions with 0 kPa, mean ± SD, *n* = 7-10 per treatment. ** Significant differences between anoxic compared to normoxic and hypoxic conditions (One-way ANOVA P < 0.0001; Bonferroni P < 0.01).

Glutathione content in tissues and hemocytes of A. islandica

The specific GSx content of mantle, gill, adductor muscle tissue and hemocytes of *A. islandica* was almost identical between normoxic and hypoxic conditions but showed tissue specific differences that ranged between 1.3 and 8 nmol mg^{-1}protein (Table 1A). Sample storage at -80°C for several months had no effect on GSx content in tissues of normoxic animals, however GSH was more oxidized after storage in the freezer, so that the GSSG content cannot be compared between freshly dissected and stored samples (data not shown). Similar specific GSx contents than in freshly dissected tissues were found in frozen mantle, gill and adductor muscle samples stored at -80°C without significant differences between bivalves incubated for 3.5 days under normoxia or anoxia (Table 1B). GSSG amounted to between 7 and 28% of the GSx determined under normoxic and hypoxic conditions (Table 1A). In gill and adductor muscle, GSSG content did not differ under both conditions, while hypoxic exposure reduced the percentage of GSSG in mantle and hemocytes by about 50% compared to normoxic exposure (Table 1A), although this difference did not reach the level of significance. Hemocytes had higher GSx contents than the other tissues under normoxia

Table 1: (A) Contents of GSx and GSSG in freshly dissected tissues and hemocytes of *Arctica islandica* that had been incubated under normoxic or hypoxic conditions and (B) contents of GSx in tissues of bivalves that had been incubated under normoxic and anoxic conditions. The latter were stored at -80°C before measurement. Data are presented as mean ± SD with *n* = 7-10 per tissue and treatment in (A) and *n* = 3-7 in (B). [a]Significant differences in GSx content of normoxic and hypoxic hemocytes compared to mantle (Two-way ANOVA $P < 0.0001$; Bonferroni $P < 0.01$). [b]Significant differences in GSSG (in % of GSx) of normoxic and hypoxic gill compared to adductor muscle (Two-way ANOVA $P < 0.0001$; Bonferroni $P < 0.05$). [c]Significant differences in GSSG (in % of GSx) in normoxic hemocytes compared to adductor muscle (Two-way ANOVA $P < 0.0001$; Bonferroni $P < 0.01$).

A	GSx (nmol mg^{-1}protein)	GSSG (nmol mg^{-1}protein)	GSSG (% of GSx)
Normoxic (21 kPa)			
Mantle	1.90 ± 0.90	0.41 ± 0.22	20 ± 9
Gill	5.51 ± 6.15	1.69 ± 2.15	26 ± 12[b]
Adductor muscle	4.82 ± 4.85	0.56 ± 0.82	10 ± 6
Hemocytes	7.94 ± 5.87[a]	1.64 ± 1.06	28 ± 16[c]
Hypoxic (2 kPa)			
Mantle	1.29 ± 0.49	0.12 ± 0.08	9 ± 4
Gill	3.20 ± 0.36	0.77 ± 0.19	24 ± 5[b]
Adductor muscle	2.68 ± 1.77	0.14 ± 0.05	7 ± 5
Hemocytes	6.09 ± 3.35[a]	0.78 ± 0.64	13 ± 8

B	GSx (nmol mg^{-1}protein)
Normoxic (21 kPa)	
Mantle	3.05 ± 0.05
Gill	9.00 ± 0.18
Adductor muscle	3.43 ± 0.34
Anoxic (0 kPa)	
Mantle	4.09 ± 1.77
Gill	10.30 ± 3.92
Adductor muscle	5.24 ± 3.87

and hypoxia, followed by GSx content in the gills, but the observed differences were statistically significant only compared to the GSx contents of the mantle. The percentage of GSSG in GSx was significantly higher in gills compared to the values found for adductor muscle under hypoxic and normoxic conditions, and in normoxic hemocytes compared to that of the adductor muscle (Table 1A).

Respiration rates of isolated gills

Gill respiration rates of *A. islandica* decreased significantly with declining oxygen concentrations in the incubation medium between 21 kPa and 2 kPa (Fig. 3). At 5 kPa gill respiration was 33 % lower and at 2 kPa 60 % lower compared to oxygen consumption rates under normoxia (Fig. 3).

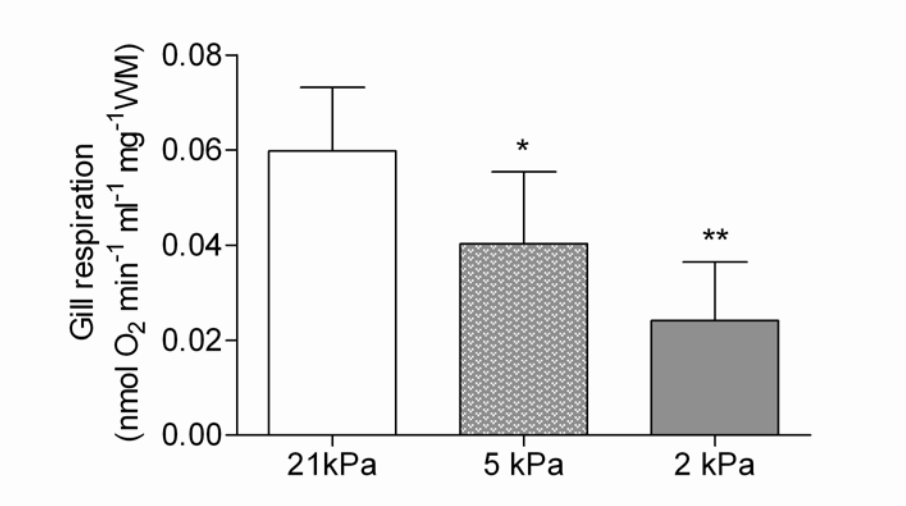

Fig. 3: Respiration rate of isolated *Arctica islandica* gill tissue incubated at normoxic PO_2 of 21 kPa, 5 kPa and hypoxic PO_2 of 2 kPa, mean ± SD, *n* = 9-16 per treatment. * Significant difference between gill respiration at 5 kPa and 21 kPa (Kruskal-Wallis $P < 0.0001$, Dunn´s $P < 0.05$). ** Significant difference between gill respiration at 2 kPa and 21 kPa (Kruskal-Wallis $P < 0.0001$, Dunn´s $P < 0.001$).

Discussion

Metabolic response to 3.5 days of hypoxia in A. islandica

Experimental hypoxia elicited behavioral and physiological responses in German Bight *A. islandica.* Siphons were permanently open at 2 kPa and respiration rates in gills decreased more rapidly below than above 5 kPa of environmental oxygen. Oxyconform respiration rates (= reduced oxygen consumption with decreasing PO_2) and a similar critical PO_2 of 4.9 kPa were already determined in respiration experiments with isolated mantle tissue of Baltic Sea *A. islandica* (Tschischka et al. 2000). This indicates that MRD sets in below 5 kPa in the ocean quahog to idle on a reduced but oxygen-based metabolic level down to extremely low environmental PO_2. Consequently, 3.5 days of hypoxic incubation did not affect the tissue levels of the anaerobic metabolites, and the specific activities of LDH or ODH were not up-regulated during hypoxic exposure. In contrast, succinate accumulated in the adductor muscle of clams exposed to the same period of anoxia. Succinate is the first intermediate which signals the onset of anaerobic metabolism in *A. islandica* muscle tissue,

and our results are in line with those of Oeschger (1990), who found around 55 nmol succinate mg^{-1}WM in the adductor muscle of Baltic Sea *A. islandica* after 3.5 days of anoxic incubation.

The reduction of metabolic oxygen demand below 5 kPa enables the ocean quahog to maintain tissues and mitochondria aerobic down to a PO_2 of at least 2 kPa. A similar response has been recorded in starved *Abra tenuis,* where direct calorimetry and respiration measurements with whole animals documented a fully aerobic metabolism under hypoxic conditions > 2.3 kPa of oxygen and the onset of anaerobic metabolism below 2.3 kPa (Wang and Widdows 1993). Permanently open siphons of *A. islandica* observed in our 3.5-day hypoxic incubation, suggest that the animals increase ventilation to remain aerobic during severe oxygen limitation. Similarly, Brand and Taylor (1974) found *A. islandica* from the Irish Sea to increase the proportion of time spent on active pumping of water to 95 % at 5 kPa PO_2, whereas at normoxic conditions each pumping period was followed by a ventilatory pause lasting for several minutes. In keeping with the better exploitation of low environmental PO_2, mitochondrial phosphorylation efficiency of White Sea *A. islandica* was found to be significantly higher at low experimental oxygen conditions of 7.3 kPa than at normoxic PO_2 (Tschischka et al. 2000). These physiological adaptations suggest that the critical PO_2 (P_{crit}) in hypoxia tolerant ocean quahog marks the onset of MRD and energy saving, rather than the compensation of oxygen deficiency by anaerobiosis, as proposed for other species (Grieshaber et al. 1994; Boutilier and St.-Pierre 2000; Boutilier 2001). We assume that shifting the onset of anaerobiosis to tissue PO_2 values far below P_{crit} forms part of the hypoxia-tolerance-strategy of *A. islandica* to survive prolonged periods of environmental oxygen deficiency.

Tissue-specific antioxidant protection at normoxic and hypoxic conditions

Our next question addressed the cellular antioxidant system and possible ROS-formation in tissues and hemocyte cells of *A. islandica* under hypoxic conditions. Metabolic depression in *A. islandica* does not only play an important role in energy reduction, it also represents a protective mechanism to slow mitochondrial ROS production and thus may be a life-prolonging strategy in the ocean quahog (Abele et al. 2010). The ratio of GSSG to GSx was lower at 2 kPa compared to 21 kPa in mantle and hemocytes, which indicates that less ROS are produced at hypoxic conditions. This is in keeping with Boyd et al. (1999), who found that ROS production rates of oyster hemocytes under hypoxia (PO_2 = 1.47 kPa) amount to only 33 % of the normoxic rates (PO_2 = 5.2 kPa).

The total GSx content in tissues of the ocean quahog indicates constant GSH redox cycling and/or synthesis of GSH during normoxia, hypoxia and anoxia. Thus no anticipatory antioxidant response to a possible reoxygenation event after environmental oxygen shortage took place. In line with this, antioxidant enzyme activities remained constant in the mantle and gill of metabolically depressed *A. islandica* after 24 h of 0 kPa in the mantle cavity water and after 3.5 days of burrowing (Strahl et al. submitted for publication). Higher levels of free radical damage during awakening and tissue reoxygenation (Ramos-Vasconcelos and Hermes-Lima 2006, Hermes-Lima and Storey 1995), as well as an up-regulation of antioxidant capacities are reported for estivating and hibernating invertebrates (Hermes-Lima and Zenteno-Sávin 2002, Bickler and Buck 2007). In contrast a ROS-burst

was absent in isolated gill tissue of Iceland *A. islandica* (Strahl et al. in revision) and of German Bight *A. islandica* (S. Hardenberg, unpublished data) after hypoxia-reoxygenation. This is possibly due to an alternative oxidase in *A. islandica* mitochondria which has a higher P_{50} than cytochrome-c-oxidase and provides a phosphorylation-independent bypass for electrons when cytochrome-c-oxidase capacities are limiting. In lowering tissue PO_2 and minimizing reduction of respiratory chain intermediates, the alternative oxidase reduces the risk of ROS formation on reoxygenation in hypoxia tolerant species (Tschischka et al. 2000). However, so far no molecular confirmation for the existence of an alternative oxidase in *A. islandica* exists. The trend of somewhat higher GSx levels in tissues of anoxia-incubated bivalves, especially in the gills, is not statistically significant and may reflect inter-individual variations in GSx.

Bivalves extract oxygen from the seawater mainly via the gill surface (Bayne et al. 1976), suggesting the possibility of higher ROS generation in the gills compared to other tissues. Further tissue respiration rates and phosphorylation efficiency are higher in gills (Strahl et al. in revision) compared to mantle of *A. islandica* (Tschischka et al. 2000), which indicate higher tissues-specific metabolic activity and explain the need for higher GSx levels and the occurrence of high GSSG amounts in the gills of the ocean quahog. Comparably high GSx contents ranging from about 0.6 to 20 nmol mg^{-1}protein were found in gill tissue of *Mytilus edulis* (Ribera et al. 1991), *Mytilus galloprovincialis* (Regoli and Principato 1995; Lima et al. 2007) and *Perna viridis* (Cheung et al. 2001).

In *A. islandica* equally high contents to gill-GSx and -GSSG were found in the hemocytes, the bivalves´ immune cells. These cells are capable of performing an oxidative burst to kill and phagocytose invading pathogens (Boyd and Burnett 1999; Pruzzo et al. 2005). In order to minimize potential oxidative damage to adjacent tissues and cells, antioxidant protection should be high in hemocyte cells. This hypothesis is in line with results reported by Pipe et al. (1993), who detected high activities of the antioxidant enzymes superoxide dismutase and catalase in granular hemocytes of *M. edulis.* Mean glutathione levels in hemocyte lysates of the scallop *Pecten maximus* were about 13.9 nmol mg^{-1}protein (Hannam et al. 2010) and, thus, comparable to the levels in *A. islandica*.

The mantle seems to be metabolically less active than other investigated tissues of the ocean quahog, because here anaerobic enzyme activities and GSx content were lowest. Significantly lower antioxidant enzyme activities in mantle than in foot muscle were also found in five different *A. islandica* populations (Basova et al. submitted), and the same was observed when comparing mantle to gill tissues in specimens of an Icelandic population (Abele et al. 2008). Interestingly, also the concentrations of the free radical damage marker lipofuscin were lower in the mantle than in the gill tissue of Iceland *A. islandica* (Strahl et al. 2007), indicating pro- and antioxidant processes to be very well balanced to meet the tissue-specific requirements.

Tissue-specific anaerobic capacities

Anaerobic capacities in *A. islandica* enable the ocean quahog to withstand extremely long periods of hypoxia and even more than 50 days of anoxia (Oeschger 1990). Octopine formation in hypoxic/anoxic adductor muscle remained low in spite of high tissue-specific ODH activity and high ODH/LDH ratio. This underlines the primary role of octopine formation during muscle work in mollusks, where it can reach concentrations of up to 9 µmol mg^{-1}WM in swimming scallops and 12 µmol mg^{-1}WM in squids (Grieshaber and Gäde 1977; Grieshaber et al. 1994). The differences in enzyme activity patterns indicate LDH and ODH to play specific roles in different tissues of *A. islandica*. Octopine produced during muscle work can be re-converted to pyruvate under aerobic conditions (Gäde and Grieshaber 1986) or it can be released into the hemolymph in order to prevent acidification of the adductor muscle. When the bivalves return to the sediment surface to take up oxygen and food, octopine originating from adductor muscle exercise may be used as an aerobic substrate in tissues with low OHD activity and low ODH/LDH ratio, such as mantle, gill and hemolymph, to feed the pyruvate produced into the Krebs cycle. Recycling of octopine and lactate instead of metabolite excretion, which was found in several marine invertebrates during the recovery process from anaerobic metabolism (Ellington 1983), could be another strategy in *A. islandica* to minimize energy loss and economize metabolism under anoxic exposure. Similarly, in tissues of the cuttlefish *Sepia officinalis* different isoforms of LDH and ODH with distinct kinetic properties coexist, which function as lactate or octopine oxidases or as pyruvate reductases (Storey 1977).

The activities of LDH in the gill and adductor or foot muscle ranged between 2 and 290 nmol/min^{-1} mg^{-1}protein in different bivalves like *M. edulis* (Long et al. 2003), *Macoma balthica*, *Scrobicularia plana*, *Cerastoderma edule*, *Mya truncata* and *Mya arenaria* (Livingstone et al. 1983) and were therefore not particularly high in tissues of *A. islandica.* For the first time, enzyme activities were not only determined in the mantle, gill or adductor muscle of bivalves but also in the hemocytes of *A. islandica,* where we found extraordinarily high LDH activity compared to the other tissues. However, at the onset of anaerobiosis, lactate formation was neither detectable in tissues and hemolymph nor in the mantle cavity and incubation water of the bivalves. Hemocyte cell numbers may just be too low to produce measurable amounts of lactate and ATP may be generated by alternative sources such as the succinate pathway. Alternatively, LDH in the hemocytes may basically function in pyruvate regeneration (see above) to keep lactate accumulation low in hypoxia- or anoxia-exposed *A. islandica*.

Our study suggests that MRD in *A. islandica* during environmental and self-induced hypoxia is an energy-saving mechanism, enabling the ocean quahog to remain fully aerobic for several days under hypoxic conditions of at least 2 kPa. Shifting the onset of anaerobiosis to tissue PO_2 values far below P_{crit} forms part of the hypoxia-tolerance-strategy of *A. islandica,* which helps to avoid the accumulation of acidic anaerobic metabolites in the cells and tissues during several days of environmental oxygen deficiency. Tissue-specific levels of glutathione (GSH), a scavenger of reactive oxygen species (ROS), indicate that tissue thiol reduction potential is maintained over extended periods of low environmental oxygen conditions, but no anticipatory antioxidant response

takes place under experimental hypoxia and anoxia exposure. Anaerobic enzyme activities are unequally distributed in bivalve tissues, suggesting specific roles of ODH and LDH as lactate or octopine oxidases or, alternatively, as pyruvate reductases (see also Storey 1977, Grieshaber et al. 1994 for review). Pyruvate reductases are known to produce metabolites (i.e. lactate, octopine, strombine and alanin) or break down metabolites, dependant on the cellular pH and the concentrations of substrates and endproducts (see also Gäde and Grieshaber 1986). Recycling of octopine and lactate instead of metabolite excretion is supposed to be a strategy in *A. islandica* to economize metabolism under anoxic exposure.

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PUBLICATION III

Metabolic rate depression: a key to longevity in the ocean quahog *Arctica islandica*

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Abstract

Arctica islandica is the longest lived non-colonial animal found so far, which reaches individual ages of 150 years in the German Bight (GB) and more than 350 years around Iceland (IC). Frequent burrowing and physiological adjustments to low tissue oxygenation in the burrowed state are discussed to extend species specific lifespan, through control of mitochondrial reactive oxygen species (ROS) formation. We investigated burrowing patterns, respiration rates and shell water PO_2 in *A. islandica* in order to determine succinate accumulation and antioxidant defences in different tissues of normoxic and metabolically depressed specimens, as well as ROS formation during hypoxia and reoxygenation. IC bivalves burrowed *in-situ* more frequently and deeper in winter than in summer, and IC and GB bivalves both remained burrowed between 1 and 6 days in laboratory experiments. Shell water PO_2 was < 5 kPa also when bivalves were maintained in fully oxygenated seawater, and ventilation increased before animals entered the metabolically reduced state. Succinate did not accumulate upon spontaneous shell closure, when shell water PO_2 was at 0 kPa for≥ 24 h. A ROS burst was absent in isolated gills during hypoxia-reoxygenation, and antioxidant enzyme activities were not enhanced in metabolically reduced compared to normally respiring clams. Avoiding anaerobiosis in the burrowed state presumably limits the need for elevated recovery respiration upon surfacing and oxidative stress during reoxygenation. An anticipatory antioxidant response during shell closure would be therefore not needed. Low shell water oxygenation and absence of a ROS overshoot when metabolism rebounds during surfacing presumably supports the enormous longevity of the ocean quahog.

Keywords

Arctica islandica, metabolic rate depression, burrowing, mantle cavity water PO_2, adenylates, antioxidative enzymes, ROS-formation, succinate

Introduction

Marine ectotherms with a wide latitudinal and geographic distribution often feature distinct population-specific maximum lifespan potentials (MLSPs). Longer MLSPs are often a consequence of cold adaptation when compared to temperate populations of the same species, or species of similar lifestyle (Brey 1991; Brey et al. 1995; Ziuganov et al. 2000; Cailliet et al. 2001; La Mesa and Vacchi 2001; Philipp 2005, Philipp et al. 2006). Extension of life expectancy in the cold presumes a delay in physiological aging, which relates to lower metabolic rates and correspondingly reduced formation of mitochondrial reactive oxygen species (ROS) in cold environments (Philipp et al. 2005; Abele et al. 2009).

The ocean quahog, *Arctica islandica* (Linnaeus 1767), is a prominent example for marine invertebrate longevity (Ridgway and Richardson 2010). Recorded MLSPs ($MLSP_{rec}$) of *A. islandica* differ regionally between populations from subarctic environments around Iceland (IC) with individual ages of more than 350 years (Schöne et al. 2005; Wanamaker et al. 2008) and from the German Bight (GB) with recorded individual MLSPs of 150 years (Witbaard and Klein 1994; Witbaard et al. 1999; Epplé et al. 2006). Low salinity populations of conspicuously shorter life expectancy have established in brackish waters of the subarctic White Sea with a $MLSP_{rec}$ of 53 years and the temperate Baltic Sea with $MLSP_{rec}$ of 40 years (Begum et al. 2010).

Arctica islandica is characterized by extremely low standard metabolic rates, slow but stable cell turnover and high antioxidant protection in cells which are maintained constant over age (Abele et al. 2008; Begum et al. 2009; Strahl and Abele 2010; Basova et al. submitted). A characteristic behaviour which might link lifestyle to mitochondrial ROS production is the self-induced hypoxia and metabolic rate depression (MRD) described for *A. islandica* from an Irish Sea population (Taylor 1976). Usually the bivalves burrow directly beneath the sediment surface and maintain fluctuating but frequently normoxic mantle cavity PO_2 by ventilation through short siphons, which are in contact with the overlying sea water. At irregular intervals ocean quahog burrow into deeper sediment horizons for periods of 1-7 days, close the shell and reduce the heart rate 10-fold compared to normoxic conditions (Taylor 1976). After several days of experimental anoxia in Baltic Sea *A. islandica,* caloric energy release is even lowered down to 1% of fully aerobic rates (Oeschger 1990). Thus, intermittent metabolic reduction could reduce lifetime ROS production in tissues of the ocean quahog and limit the oxidative damage levels compared to other bivalves (see protein carbonyl comparison in Abele et al. 2008).

A problem with burrowing and self induced hypoxia or anoxia is that this behaviour could cause oxidative stress when the bivalves surface again, similar to hypoxia-reoxygenation injury in humans (Li and Jackson 2002). However, in several hypoxia- and anoxia-tolerant vertebrates and invertebrates that undergo estivation, hibernation or freezing, MRD appears to trigger antioxidant functions, presumably to reduce hypoxia-reoxygenation injury during emergence from MRD (Boutilier

and St-Pierre 2000; Hermes-Lima and Zenteno-Savín 2002; Lushchak et al. 2005; Larade and Storey 2009). The question is, whether similar mechanisms control oxidative stress in surfacing *A. islandic*a. Both, MRD-duration (in days) and MRD frequency, as well as the regulation of antioxidant capacities in *A. islandica* might depend on environmental temperature, salinity and/or food availability and thus differ between populations and may differentially modulate MLSP in different regions.

If metabolic depression is the key to a long life expectancy in *A. islandica*, it should be self-induced, voluntary and accompanied by energy saving mechanisms in order to avoid considerable accumulation of acidic anaerobic metabolites (i.e. succinate, lactate, opines and short chained organic acids). The ocean quahog should have evolved strategies to balance ATP-supply and demand, and stabilize tissue energy charge (EC) during MRD. Earlier works confirm that *A. islandica* features high capacities of anaerobic ATP-production and that mitochondrial anaerobic pathways are used to maximize the yield of ATP formed per mol of H^+ during low oxygen periods (Livingstone et al. 1983; Oeschger 1990; Oeschger and Storey 1993; Strahl et al. 2011). This enables the bivalves to survive extended periods of oxygen deficiencies caused by eutrophication in the North Sea and the Baltic Sea (Rosenberg et al. 1992; Oeschger and Storey 1993; Diaz and Rosenberg 1995).

The complications in surviving hypoxia reoxygenation and energetic limitations in burrowed clams might be ameliorated at low temperatures, and the question arises to what extent the longer lifespan of the IC population is supported by the permanently cold climate. To answer this question, we studied burrowing behaviour of IC and GB *A. islandica* of similar shell size (shell height IC clams: 37 – 90 mm; GB clams: 68 – 84 mm) in the laboratory at 10 °C water temperature, well within the temperature window of both populations. The seasonality of burrowing was investigated *in-situ* in an IC population. PO_2 measurements in the mantle cavity water of GB specimens were made with implanted needle optodes, to investigate whether the animals expose their tissues to hypoxia/anoxia voluntarily in a normoxic environment. ROS-production rates were determined in isolated gill tissue at normoxia, hypoxia and after hypoxia-reoxygenation. Tissue samples of GB and IC *A. islandica* from the burrowing experiments and the PO_2 measurements were analyzed for antioxidant enzyme activities, total adenylates and citrate synthase activity, as well as for accumulation of the anaerobic metabolite succinate.

Materials and Methods

Arctica islandica were sampled from two geographically separated populations (Fig. 1). In laboratory and field experiments, investigations of the burrowing behavior and self-induced MRD live *A. islandica* were conducted (Fig 1). Bivalves used in the laboratory experiments, were subsequently dissected and tissue samples of individuals were either directly analyzed or snap-frozen in liquid nitrogen for further physiological analysis (Fig 1).

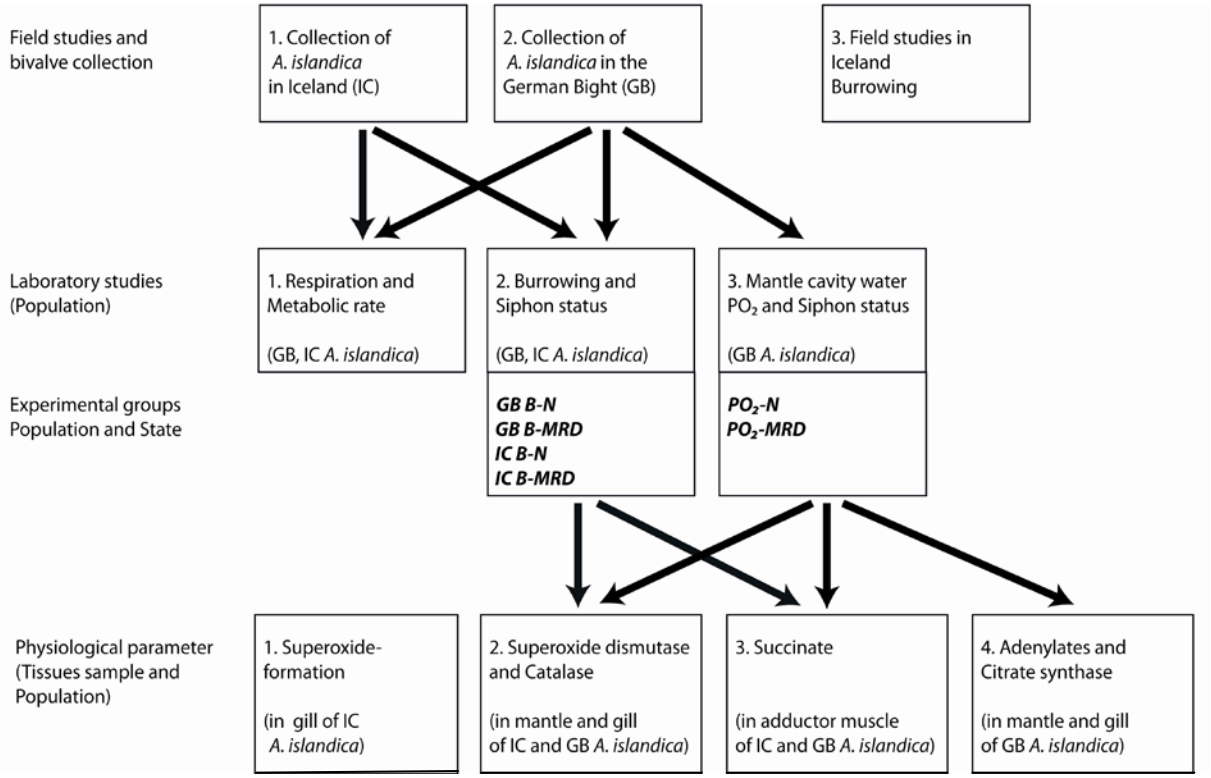

Fig. 1: Flow diagram of field work, laboratory experiments and measurements of physiological parameters in *Arctica islandica* from Iceland (IC) and the German Bight (GB). Experimental bivalves were either non-burrowed (*B-N*) or 3.5-days-burrowed (*B-MRD*), and in a second experimental approach of changing PO_2 in the mantle cavity water (*PO_2-N*) or of ≥ 24 h 0 % O_2 in the mantle cavity water (*PO_2-MRD*), *n* = 9-11.

Field study

In June 2003 and February 2004 burrowing activity of *Arctica islandica* was determined by divers in-situ in Eyjafjördur, North Iceland (65°47.86'N, 18°3.76'W, Fig. 1, 2) at 10 m depth. The seafloor at the site of investigation consists of medium grained sand with 0.25-0.49 mm grain size. Sea water temperatures were not recorded during the field study, but average monthly surface water temperatures in Eyjafjördur between 1987 - 2000 ranged at 7.5°C in June and 1.5°C in February (Jónasson 2004). Average phytoplankton concentrations, measured in the years 1992 and 1993 (Kaasa and Gudmundsson 1994) at a nearby locality (2.5 km away), were 0.03 ± 0.01 mg chl m^{-3} in February and 0.9 ± 0.4 mg chl m^{-3} in June.

On each sampling date, divers positioned 1 square meter frames on the seabed and collected all clams of >10mm shell length individually using an underwater suction sampler. *Arctica islandica* has two short siphons, and its burrow openings appear as paired cylindrical holes. When the siphon openings were visible at the sediment surface the burrowing depth was considered 0 cm. In deeper burrowed clams, where siphons were invisible, the depth of burial was measured with a ruler to the nearest 0.5 cm and was recorded as the distance from the seafloor surface to the uppermost part of the clam. Ocean quahog were encountered as deep as 12 cm.

Bivalve collection and maintenance

Arctica islandica were collected in May 2008 at Helgoland 'Tiefe Rinne' in the German Bight (54°09.05'N, 07°52.06'E, Fig. 1, 2) at 40−45 m water depth, using a trawl net. Surface water temperature was 12°C. In August 2008 *A. islandica* were collected northeast of Iceland (66°01.44'N, 14°50.91'W, Fig. 1, 2) at 8−15 m water depth at a surface water temperature of 9°C. Bivalves from the German Bight (GB) and Iceland (IC) were transported in cooled containers to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven. *Arctica islandica* were acclimated for two weeks at 10°C and 33 PSU in 60-l aquaria with re-circulating sea water containing 10 cm of pea gravel sediment of 2-3 mm grain size and fed once a week with a mixture of *Nannochloropsis occulato*, *Phaeodactylum tricornutum* and *Chlorella sp*. (DT´s plankton farm, USA, 3 ml bivalve^{-1} wk^{-1}).

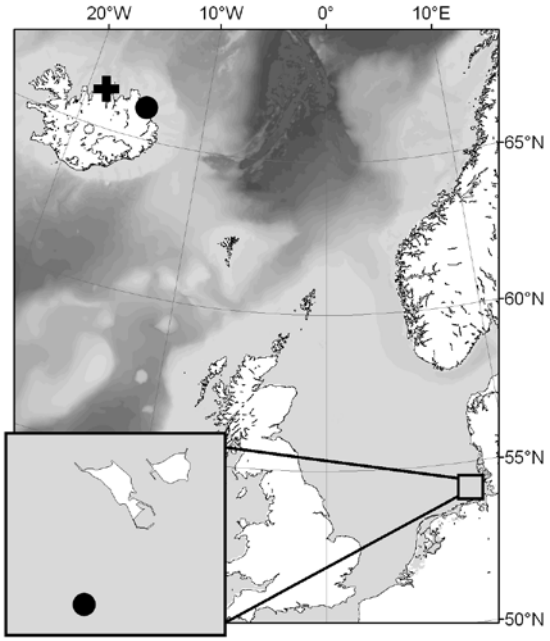

Fig. 2: Location of the field study (black cross) north of Iceland and sampling locations (black circles) of experimental *Arctica islandica* near Iceland (mean sea surface temperature (SST): 5°C, annual range: 2-10°C, 34 PSU, http://www.hafro.is/Sjora) and the German Bight near Helgoland (see frame insert, SST: 9°C, annual range: 4-19, 34 PSU, http://www.bsh.de/de/Meeresdaten/ Beobachtungen/ MURSYS-Umweltreportsystem/index. jsp). Continental shelves appear light gray, deeper areas are shaded darker; source of bathymetry: Smith and Sandwell (1997).

Laboratory burrowing experiment

Wire straps of 18 cm length and 0.3 cm diameter were numbered and attached to shells of 30 IC and 30 GB *A. islandica* next to the siphon-openings with epoxy adhesive glue (Reef construct, Aqua medic, Bissendorf, Germany), which solidified completely underwater within 24 h. Clams were kept at 10 °C in 60-l aquaria containing 20 cm of pea gravel sediment. Daily burrowing depth of each clam was determined to the nearest 0.5 cm by measuring the length of the wire which was visible above the sand (Fig. 1). GB and IC *A. islandica* which were burrowed for 3.5 days in more

than 3 cm sediment depth were sampled as MRD animals (*B-MRD*, Fig. 1). Bivalves that showed their siphons open for 3.5 days at the sediment surface were sampled as normoxic control animals (*B-N*, Fig. 1). Ten individuals of each population and status were dissected and mantle, gill and adductor muscle were snap frozen in liquid nitrogen for the analysis of physiological parameters. Shell height of IC clams ranged from 37 to 90 mm and of GB clams from 68 -84 mm.
Additionally the siphon-status of another 10 GB and 10 IC *A. islandica* was documented individually for 2 weeks using a video camera (Panasonic NV-GS 180) and the VisionGS Software (Business edition-V1.51 test release). Every ten minutes a snapshot of the siphon-opening of one clam was taken and stored for further analysis (Fig. 1).

Oxygenation (PO_2) of the mantle cavity water

The PO_2 in the mantle cavity water of GB *A. islandica* was measured to clearly identify individuals which underwent MRD. Bivalves with a recorded permanent $PO_2 = 0$ for ≥ 24 h were identified as MRD-animals (*PO_2-MRD*, Fig. 1). Contrary, in normoxic, ventilating clams (*PO_2-N*, Fig. 1) the PO_2 in the mantle cavity water frequently fluctuated between 100% and 0% PO_2. Measurements of PO_2 were carried out using single channel Microx TX-3 oxygen meters equipped with oxygen needle-optodes (12 x 0.04 mm, PSt1-L5-TF) of PreSens (Precision Sensing GmbH, Regensburg, Germany) that had fluorescent coated flat tips of 140 µm diameter. Prior to the measurements the optodes were calibrated to 100% air saturation with aerated seawater and to 0% using water saturated with nitrogen at 10°C. *Arctica islandica* were kept in fully aerated seawater of 10°C and 33 PSU in 20-l aquaria, which were laminated with black foil and covered during the experiments to minimize disturbances of the clams by movements in the laboratory. In order to avoid damage of the optodes, bivalve mobility was restricted. At least 24 hours before an experiment, a teflon nut was glued to the bivalve's lower shell to fix the animals with a screw to an experimental platform in the aquarium (see also Abele et al., 2010). A 1 mm hole was drilled into the shell about 1 cm from the edge, which was covered with thin elastic latex foil (Rubber Dam, Heraeus Kulzer, Germany) and isolation material (Armaflex, Armacell, Germany), to avoid exchange between mantle cavity water and aquarium water. One hour before starting the oxygen measurements, a hole was pinched through the isolation material and the optode was gently introduced into the mantle cavity. Air saturation was recorded in 30 second intervals using the TX3_v520 software of PreSens. Additionally, the siphon-status of each experimental clam was documented during oxygen measurements by taking 10-min-snapshots of the siphon-opening with a video camera placed outside the aquaria, where part of the black foil was removed (Fig. 1). Specimens that maintained zero PO_2 for 24h were taken as *PO_2-MRD*-animals by definition (see above). PO_2 measurements were terminated and the clams were dissected and mantle, gill and adductor muscle tissue snap frozen in liquid nitrogen. Bivalves that did not experience prolonged periods of zero PO_2 in extrapallial fluid (*PO_2-N* bivalves) were also dissected and tissues snap frozen in liquid nitrogen. Oxygen saturation data (%) were converted to oxygen partial pressure (kPa) and to frequencies corresponding to

1 kPa classes ranging from class 0 kPa to 21 kPa according to Abele et al. (2010). Nine *PO_2-MRD*- and nine *PO_2-N*-animals with a shell height between 73 and 87 mm were analyzed and processed.

Respiration and metabolic rate

Respiration rates of GB and IC *A. islandica* (Fig. 1) were measured separately at 10°C in a multichannel intermittent flow system as described by Begum et al. (2009) and Heilmayer and Brey (2003). To avoid an influence of specific dynamic action (SDA) on respiration, bivalves were maintained without food for three days (Bayne et al. 1976), and measurements were started after one night acclimation of the animals in the respiration chamber. Oxygen concentration was monitored continuously with fluorescein-coated oxygen needle-optodes connected to a MICROX TR3 array. Following the measurements, clams were dissected and soft tissue dried for 50 h at 68°C to determine dry mass (DM). To obtain ash free dry mass (AFDM = DM - ash), the dried tissues were combusted for 24 h at 50°C. Individual oxygen consumption rate (VO_2, µmol O_2 h^{-1} ind^{-1}) and mass specific respiration rates (MSR, µmol O_2 h^{-1} ind^{-1} $AFDM^{-1}$) were calculated according to Begum et al. (2009). Experimental animals that did not consume oxygen during 24h in the respiration system were substituted by other test specimens. 13 GB *A. islandica* with a shell height between 69 and 77 mm and 16 IC *A. islandica* with a shell height between 45 and 90 mm were measured.

ROS-formation in isolated gill tissue

ROS-formation was measured with the redox-sensitive, cell-permeable fluorophore Dihydroethidium (DHE), which directly detects cellular superoxide production (Fig. 1) *in vivo*, modified after Kalivendi et al. (2003). DHE is oxidized by superoxide with the substrate entering the nucleus and binding to DNA, thus enhancing fluorescence only in the nucleus (Zhao et al., 2003). IC *A. islandica* were kept without food for three days before starting the experiments. Bivalve gills were dissected and small gill pieces of 2 or 3 filaments were excised and transferred to incubation vials filled with incubation buffer (450 mM NaCl, 10 mM KCl, 20 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 0.5 mM DTT, 0.055 mM glucose, pH 7.4) cooled to 8°C and of varying PO_2. One part of the filamentous tissue was incubated immediately after dissection for 20 min in 6 ml incubation buffer with 10 µM DHE at 21 kPa to measure ROS formation in the freshly excised tissue. Two pieces of similar weight were incubated for 2 h in 6 ml incubation buffer at 21 kPa or 5 kPa, respectively. The hypoxic PO_2 was adjusted to 5 kPa by using a Wösthoff gas mixing pump and mixing oxgen and nitrogen gas (Wösthoff GmbH, Bochum, Germany). After 2 h of incubation, 10 µM DHE were added directly to incubation medium at 21 kPa or 5 kPa, respectively. After 20 min the gill filaments were washed in clean buffer, transferred to a microscopic slide and bathed in clean buffer to determine the superoxide-production during the 20 min-incubation. For measurements under hypoxia, filaments were washed and bathed in incubation buffer at 5 kPa. Hypoxic conditions were maintained during the measurements by closing the chamber with a cover slip. In order to determine superoxide production under hypoxia reoxygenation, a fourth part of filaments was incubated

for 2 h at 5 kPa and reoxygenated for 20 min after adding 10 µM DHE. Chambers were cooled to 4°C using a refrigerated microscope stage connected to a thermostat (Ecoline RE 106, Lauda, Germany), to avoid radical formation in the gill filaments due to artifactual warming. Fluorescence images were obtained with a fluorescence microscope (Leica TCS, Germany) at λ_{ex} 488 nm and emission at 560/60 nm. To evaluate ROS production, the ratio of stained area of the total area in defined image sections was calculated using the software ImageJ (Version 1.43U, National Institutes of Health (NIH), USA).

Superoxide dismutase activity

Superoxide dismutase activity (SOD, Fig. 1) was determined after Livingstone et al. (1992). 100–180 mg of frozen mantle and gill tissue were ground in liquid nitrogen and homogenized with a micropistill in Tris-HCl buffer (20 mM TRIS, 1 mM EDTA, 20 mM HCl, pH 7.6) 1:10 (w/v). Samples were centrifuged for 3 min at 18 000 *g* and 4°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, 20 °C. One unit SOD causes 50% inhibition under assay conditions. Mitochondrial and cytosolic SOD isoforms were not distinguished.

Catalase activity

Catalase activity (CAT, Fig. 1) was determined after Aebi (1984). 20–50 mg of frozen mantle and gill tissue were ground in liquid nitrogen and homogenized 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 1:30 (w/v). Samples were centrifuged for 15 min at 13 000 *g* and 4°C, and CAT activity was determined by recording the period required for H_2O_2 decomposition at 20 °C, resulting in a decrease of absorption from 0.45 to 0.4 at 240 nm (1 unit).

Adenylate concentrations and energy charge

Concentrations of ATP, ADP and AMP (Fig. 1) were determined after Lazzarino et al. (2003), using high performance liquid chromatography (HPLC). Frozen samples of mantle and gill were ground in liquid nitrogen and homogenized with a micropistill in nitrogen-saturated precipitation solution (CH_3CN [Acetonitril] + 10 mM KH_2PO_4 at a 3:1-ratio, pH 7.4) at 1:11 (w/v). Samples were centrifuged for 10 min at 20 690 g and 4°C and clear supernatants were stored on ice. Pellets were resuspended in 1 ml precipitation solution using an ultra-turrax (IKA-Werke, Germany) for 5 sec and centrifuged for 10 min at 20 690 g and 4°C. Secondary supernatants were combined with primary, washed with the double volume of chloroform (CH_3Cl HPLC grade) and centrifuged for 10 min at 20 690 g and 4°C. The upper aqueous phase contains the water-soluble low molecular weight compounds and was washed twice with chloroform, centrifuged and stored at -80°C. Samples were defrosted, centrifuged for 20 min at 20 690 g and 4°C and measured by HPLC using a Kromasil 250 x 4.6 mm, 5 µm particle size column (Eka Chemicals, AB, Sweden) and a guard

column. 50 µl of samples were injected and HPLC conditions such as solvents, gradients, flow rate and detection were applied according to Lazzarino et al. (2003). Adenylate standards were purchased from Sigma-Aldrich (Steinheim, Germany). The total amount of adenylates (= ATP + ADP + AMP) was added and energy charge was calculated from adenylate according to Atkinson (1968) cf. Ataullakhanov and Vitvisky (2002) using Karat Software 7.0 (Beckmann Coulter, Germany):

$$EC = \left[ATP + \left(ADP/2\right)\right]/\left(ATP + ADP + AMP\right)$$

Citrate synthase activity

The activity of the mitochondrial key enzyme citrate synthase (CS, Fig. 1) was determined after Sidell et al. (1987). Frozen samples of mantle and gill tissues were ground in liquid nitrogen and homogenized with a glass homogenizer (Nalgene, USA) in Tris-HCl buffer (20 mM Tris-HCl, 1 mM EDTA, 0,1 % Tween®20, pH 7.4) at 1:10 (w/v). Samples were sonicated for 15 min at 2°C in a Branson Sonifier 450 (Duty cycle 50%, output control 8) and centrifuged for 5 min at 7400 g at 4°C. CS activity was measured at 20 °C by recording the absorbance increase of 5 mM DTNB (5,5´dithiobis(2-nitrobenzoic acid)) in 75 mM Tris-HCL (pH 8.0), 0.4 mM Acetyl-CoA and 0.4 mM oxalacetate at 412 nm. CS activity was calculated using the mmolar extinction coefficient ε_{412} of 13.61 $mM^{-1}\,cm^{-1}$.

Succinate concentration

Frozen samples of the adductor muscle were homogenized on ice using an ultra-turrax and ultrasound (Bandelin, Germany) in 0.5 M perchloric acid (PCA) at 1:6 (w/v) and centrifuged for 15 min at 12 000 g and 4°C. The supernatant of each sample was neutralized with a predefined amount of 2 M KOH and centrifuged for 5 min at 12 000 g and 4°C. The succinate content (Fig. 1) was determined after Michal et al. (1976) using the succinic acid Assay kit (Cat. No. 10 176 281 035, Boehringer Mannheim/R-Biopharm, Germany). The absorbance of NADH was recorded in UV-DU 800 spectrophotometer (Beckmann, Germany) at 340 nm and 37°C. The incubation time was prolonged to 30 min for complete enzymatic reaction of the succinate in the sample.

Statistical analysis

All data sets were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett´s test) before statistical analysis. Burrowing behaviour of *A. islandica* in the field may be affected by season, PO_2 in mantle cavity water by siphon status, and mass-specific respiration rates by population. Thus the effects of season, siphon status and population were tested by T-test (Mann Whitney test for non-Gaussion distributed data). The siphon status, superoxide-formation, enzyme activities (SOD, CAT CS) as well as adenylate and succinate content may be affected by population, experimental PO_2, tissue type, and/or status (N vs. MRD). We tested for differences between populations, experimental PO_2, tissue type and status by ANOVA (Kruskal-Wallis test for non-Gaussian distributed data) and post-hoc test Tukey (Dunn´s).

Effects of status and exposure time on mantle cavity water PO_2

We reduced temporal resolution of each individual PO_2 time series from the original 30 sec to 5 min by averaging the measurements over subsequent 5 min intervals (i.e. 12 values per interval). All individual time series were reversed in order to start the comparison from a common endpoint and cut to the period 0 - 2350 min to obtain equal time series length for all *PO_2-N*- and *PO_2-MRD*-clams. The pooled PO_2 data were Box-Cox transformed (Sokal and Rohlf 1981) to meet preconditions of ANCOVA and tested for effects of STATUS (*PO_2-N*- and *PO_2-MRD*-clams), TIME and BODY MASS (shell free wet mass) by a fully factorial analysis of covariance model (ANCOVA, i.e. $PO_{2,BoxCox}$ versus STATUS and covariates TIME and BODY MASS). As all parameters and the STATUS interaction terms were found to affect PO_2 significantly (see results), we used the residuals of the ANCOVA model to check for status specific temporal patterns in PO_2. Visual inspection of the residuals versus time plots indicated distinct differences in temporal development of mantle cavity PO_2. Accordingly, we compared the time intervals 100 - 2350 min in *PO_2-N* clams, and 800 - 2350 min as well as 100 - 800 min in the *PO_2-MRD* clams by means of one-way ANOVA with subsequent Tukey post-hoc test on differences between means.

Endogeneous rhythms in mantle cavity water PO_2

From the 18 *PO_2-N* and *PO_2-MRD* individual time series produced, we selected those 13 series with at least 450 consecutive 5 min data points and not more than one gap (malfunction of optode) in the time series. These gaps (3 series) were closed by linear interpolation. We removed the long term trend (across >100 data points) from each time series by means of cubic spline interpolation (settings: shape parameter $\lambda = 10000$). The time series of the residuals of the cubic spline interpolation was taken as representative for short-term variability in mantle cavity PO_2. Correlation analysis of the residual time series failed to detect any significant positive correlation between the specimen no. 2, 3 and 4 that had been measured simultaneously, i.e. there was no external forcing of the oscillations in PO_2 detectable. We analyzed each PO_2 time series by a two-step procedure using the software package kSpectra (SpectraWorks Inc, USA). In the first step we applied Singular Spectrum Analysis (SSA; settings: window length 60, covariance estimation by the approach of Vautard and Ghil (1989), Monte Carlo significance test) to identify the strongest oscillatory components. Ranked by variance explained, the first ten SSA components (singular values) captured close to 50% of total variance in each time series. These ten components were used to reconstruct a 'filtered' PO_2 time series. In the second step, the reconstructed PO_2 time series was subjected to the nonparametric Multi-Taper method (MTM; settings: significance = 'red noise', 3 tapers, adaptive procedure, robust background noise) of spectral analysis which is a common tool in geophysics, oceanography, climatology and geochemistry (Mann and Lees, 1996). Within the frequency range zero to 0.5, spectral density is computed for 512 equally spaced frequencies. See Ghil et al. (2000) for more detailed information on SSA and MTM. The resulting 512 frequencies x 13 individuals matrix was subjected to Principal Component Analysis (on the covariance matrix) and the first principal component was taken to represent the common spectral pattern of all 13 time series.

Results

Burrowing behaviour, siphon status, measurement of the mantle cavity water PO_2, whole animal respiration and ROS-formation in isolated gill tissue of GB and IC Arctica islandica

In-situ burrowing depth of IC *A. islandica* differed significantly between winter and summer (Fig. 3). In February 2004, *A. islandica* were found in 4 - 12 cm sediment depth (mean = 8.5, s.d. = 1.7), whereas in June 2003 clams were found in 0 – 10 cm sediment depth and were, on average, significantly closer to the surface (mean = 2.4, s.d. = 2.2, Fig.3).

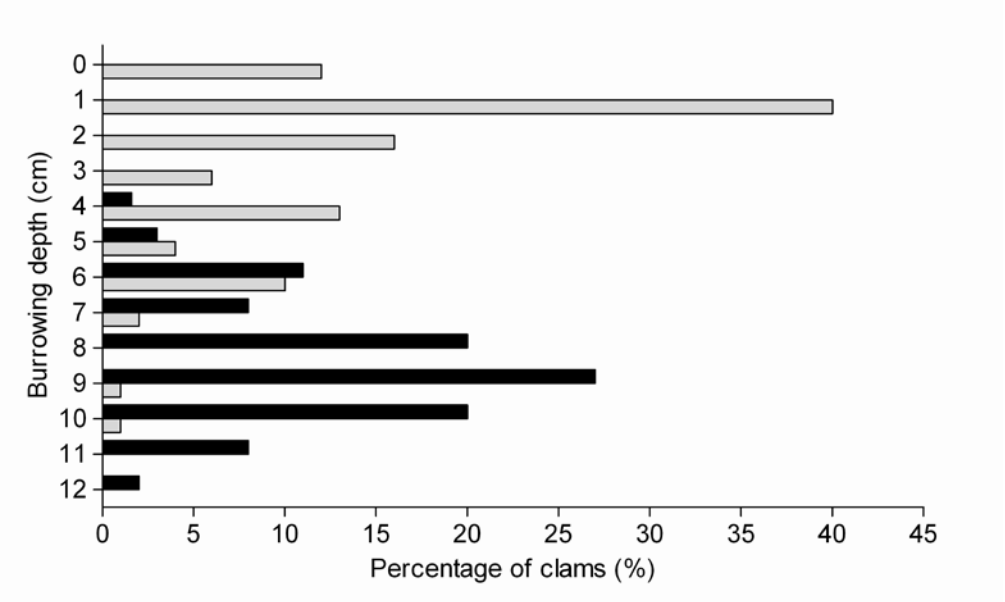

Fig. 3: Percentage of *Arctica islandica* in Iceland seabed studies burrowed at different sediment depth in June 2003 at average monthly SST of 7.5 °C (grey bars, *n* = 111) and in February 2004 at SST of 1.5 °C (black bars, *n* = 79).

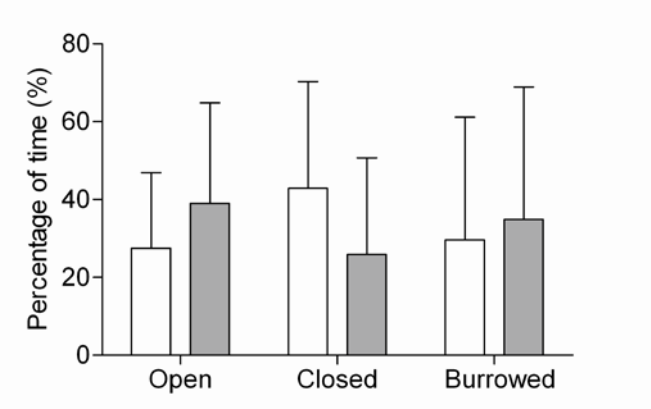

Fig. 4: Siphon-status of *Arctica islandica* from the German Bight (open bars, *n* = 23) and Iceland (grey bars, *n* =9) filmed in laboratory tanks, mean ± s.d., experimental temperature = 10 °C.

The 'burrowing and siphon status' experiment (Fig. 1, Laboratory studies No 2) indicated no significant differences in siphon activity (open/closed) or burrowing status between GB and IC animals (Fig. 4). The exact burrowing depth of the clams was not determined in the experiment, but periods

during which clams remained constantly burrowed lasted equally long, between 1 and 6 days, in both populations.

PO_2 measurements in the mantle cavity water of GB *A. islandica* showed that the clams actively regulated oxygen concentrations when deprived of their sedimentary retreat (Fig. 5). Ventilation behaviour differed between individuals, and mantle cavity PO_2 fluctuations were strong and fast in some clams and less pronounced in others (individual recordings are not shown). The mean PO_2 during the intervals where siphon closure was confirmed by photographic recording in *PO_2-N-* and *PO_2-MRD*-clams, prior to entering the MRD status, was 4.03 ± 5.27 kPa (*n*=9) and significantly lower than during siphon-opening, 4.96 ± 6.29 kPa (*n*=9) (t-Test $P < 0.0001$). The frequency distribution of recorded PO_2 values (5 min averages) over kPa classes from 0 kPa to 21 kPa (=100 %) indicated that the PO_2 in the mantle cavity water was zero about 25 % of all observation time, whereas PO_2 values ≥ 14 kPa were recorded during 16 % of all time (Fig.5).

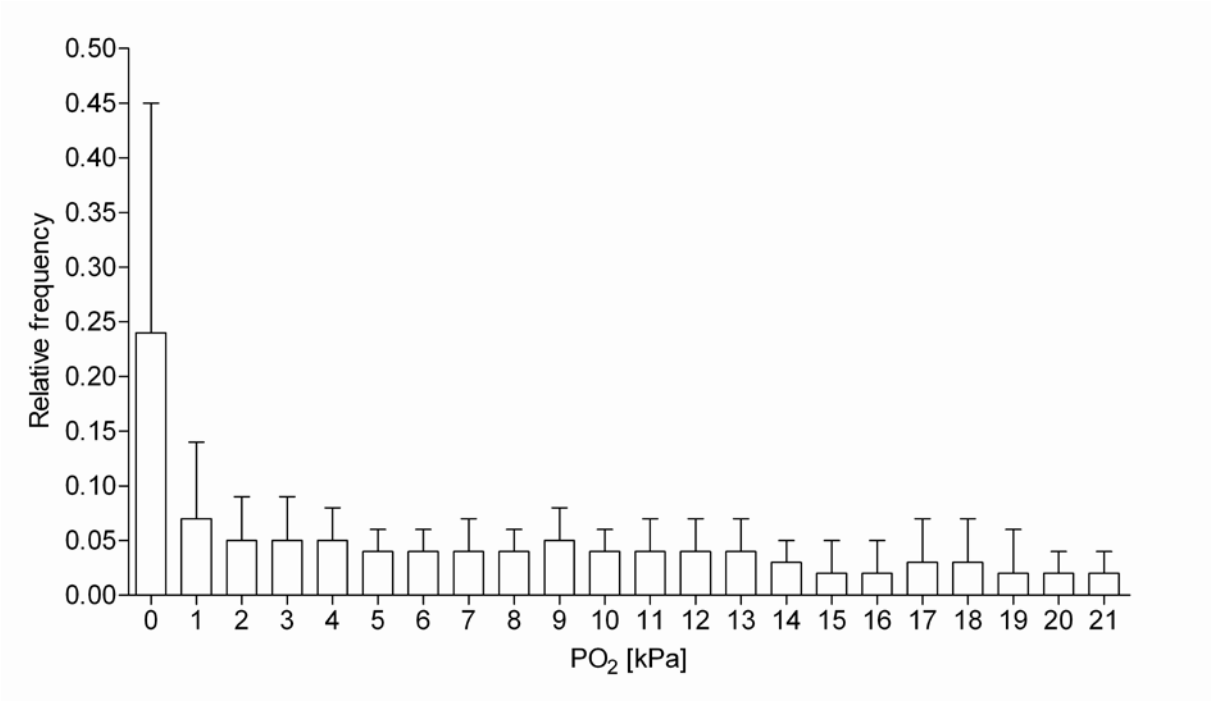

Fig. 5: Mean relative frequencies of PO_2 in the mantle cavity water of *Arctica islandica* from the German Bight across 1 kPa classes (21 kPa = 100 % saturation). Values include all PO_2 measurements of normoxic clams (*PO_2-N*) and of *PO_2-MRD*-clams prior to MRD status (defined as PO_2 = 0 for≥ 24 h in the mantle cavity water), *n* = 18, mean ± s.d., experimental temperature: 10 °C.

Mantle cavity water PO_2 was significantly affected by STATUS, TIME, BODY MASS and the interaction terms STATUS X TIME and STATUS X BODY MASS (ANCOVA, $P < 0.0001$ for all terms). The residuals of this model revealed a distinctly different behaviour of PO_2 in *PO_2-MRD* clams, particularly during the last 700 min prior entering MRD. In *PO_2-MRD*-clams, PO_2 was significantly lower than in *PO_2-N*-clams (100 - 2350 min, mean residuals = -0.2115 ± 0.072) when animals were more than 700 min away from the MRD status (800 - 2350 min, mean residuals =-0.541 ± 0.089, Tukey

P < 0.001), but significantly higher in the 100 - 800 min interval directly before entering the MRD status (mean residuals = 1.918 ± 0.133, Tukey P < 0.001, Fig.6A, B).

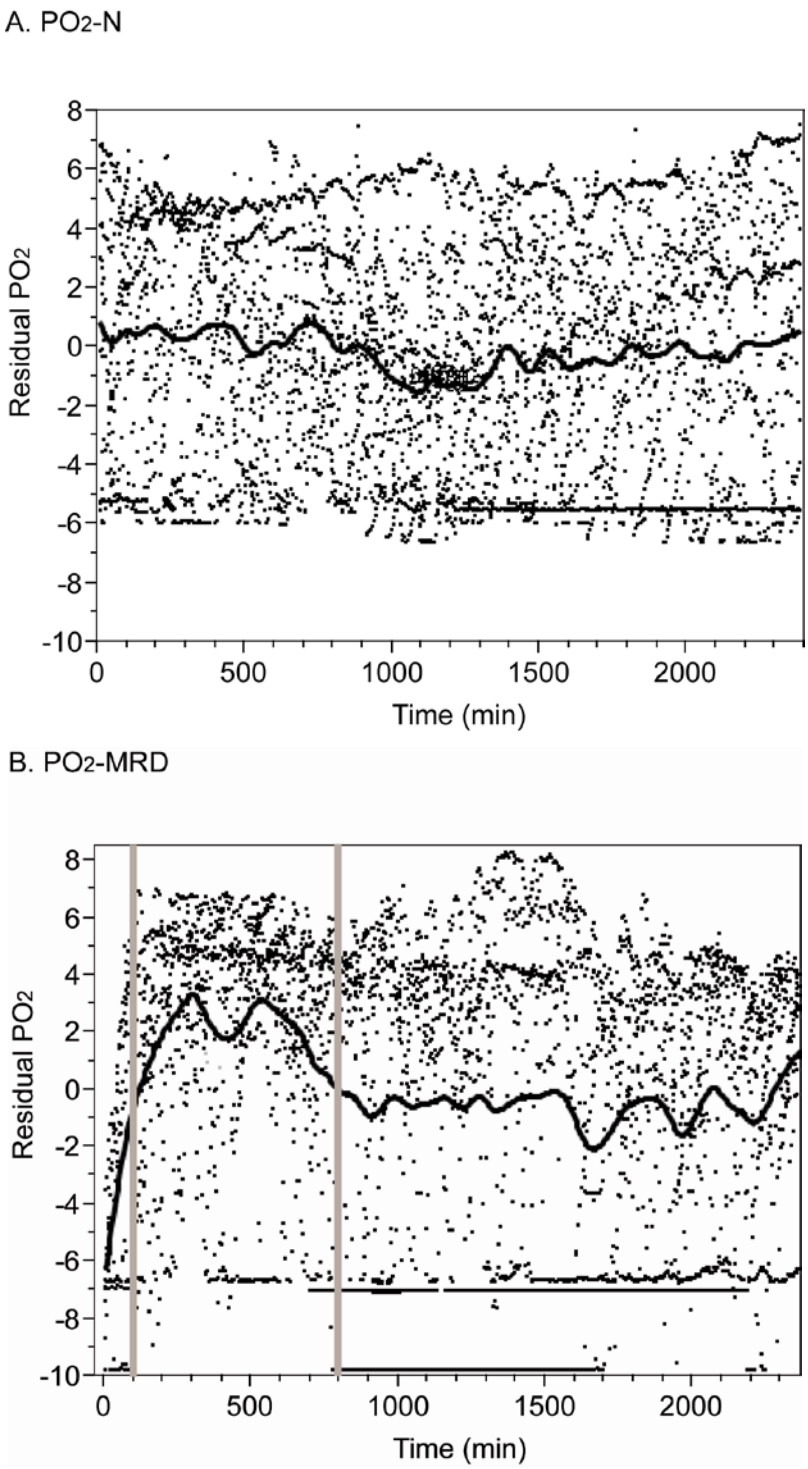

Fig. 6: Residuals of the ANCOVA model plotted over time for (A) normox (PO_2-*N*) *A. islandica* (*n* = 8) and (B) PO_2-*MRD A. islandica* prior to the MRD status (defined as PO_2 = 0 for ≥ 24 h in the mantle cavity water, *n* = 8) from the German Bight, experimental temperature = 10 °C. The black line represents the cubic spline interpolation ($\lambda = 10^6$). Time axis is reverse, zero represents the end of the oxygen measurements in (A) and the onset of MRD in (B). Grey bars in (B) indicate the 100-800 min time window with specific pre-MRD PO_2 development.

Spectral analysis revealed a prominent signal at frequency 0.1 $5min^{-1}$, i.e. the 13 clams analyzed exhibited a common oscillatory pattern in mantle cavity water PO_2 with a period of about 50 min (Fig.7).

Mass specific respiration rates at 10°C were significantly higher in IC compared to GB *A. islandica* (Table 1). A similarly high percentage of IC and GB clams of more than 30 % did not respire for 24 h (Table 1).

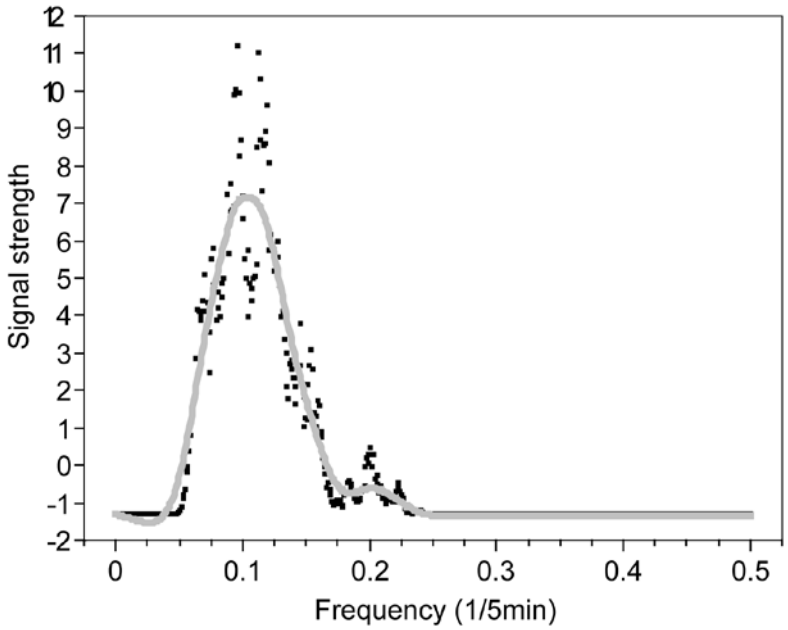

Fig. 7: 1st axis of the Principal Component Analysis of the 13 spectra of German Bight *Arctica islandica* with superimposed cubic spline fit ($\lambda = 10^{-7}$), major peak at F = 0.1 (period 50 min), experimental temperature = 10 °C.

Table 1: Mass specific respiration rate (MSR) standardized to mean size mollusks of 3.54 g AFDM at 9 °C in German Bight and Iceland *Arctica islandica*. Data in mean ± s.d., *n* = 8-32, experimental temperature = 10 °C. (1) MSR-data are taken from Basova et al. submitted. * Significant differences between clams of the two populations (Mann Whitney $P < 0.001$). Quahogs which did not respire for 24 h are given in number and in % of all investigated bivalves.

	MSR	non-breathing *A. islandica* (in %)
German Bight	2.43 ± 0.38(1)	5 of 13 (39 %)
Iceland	3.97 ± 1.72(1) *	5 of 16 (31 %)

Superoxide formation in the isolated gill tissue was highest directly after animal dissection. ROS-signal intensities were significantly lower after 2 h at 5 kPa (hypoxia) compared to values in freshly excised (FE) tissues or control tissue pieces from the same animal maintained 2 h at 21 kPa. After 2 h at 5 kPa with subsequent 20 minutes of reoxygenation (21 kPa), the signals of superoxide formation were slightly higher than in hypoxic incubated tissues, but still significantly lower than in FE tissues and 4-fold lower than after 2 h at 21 kPa (Fig. 8).

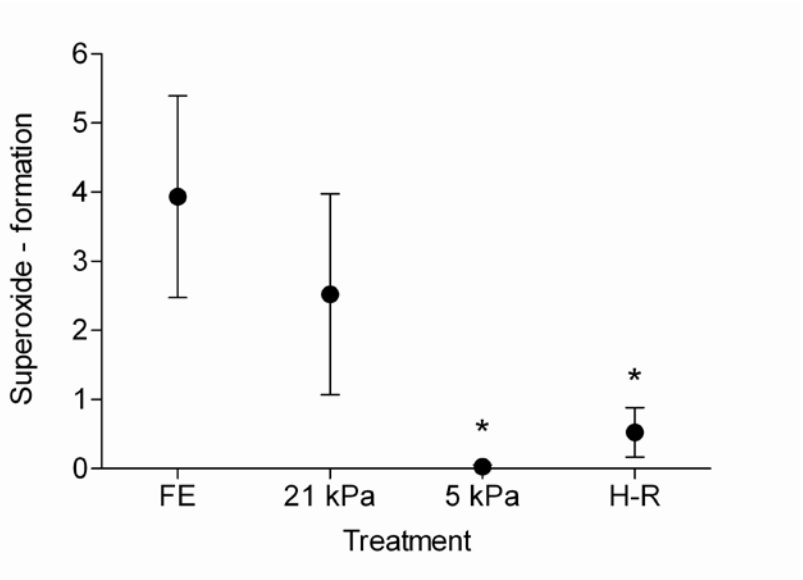

Fig. 8: Superoxide formation (in % stained after 20 min of incubation with DHE) in gill tissues of IC *Arctica islandica* directly following dissection (FE, n = 5), after 2 h of incubation under normoxic Po_2 conditions (21 kPa, n = 3) or hypoxic Po_2 conditions (5 kPa, n = 3), or after hypoxia-reoxygenation (H-R, n = 11), mean ± s.d., incubation temperature = 8 °C. * Significant difference between 5 kPa *vs*. FE and 21 kPa; and significant difference between H-R and FE (Kruskal-Wallis $P < 0.001$, Dunns $P < 0.05$).

Antioxidant enzyme activity, citrate synthase activity, content of adenylates in mantle and gill and of succinate in the adductor muscle of GB and IC Arctica islandica

In mantle and gill no significant differences were found in catalase (CAT) and superoxide dismutase (SOD) activity between *PO_2-N* and *PO_2-MRD* clams and between *B-N* and *B-MRD* clams of both GB and IC *A. islandica.* Nevertheless, enzyme activities in tissues of *B-MRD* clams were mildly but consistently higher than in *B-N* clams (Table 2). SOD activity was similarly high in mantle and gill of GB and IC *A. islandica.* CAT activity of IC clams was significantly higher in gills than in mantle, whereas in GB *A. islandica* no tissue specific differences were found (Table 2).

SOD activity was similarly high in mantle and gill of GB and IC *A. islandica.* CAT activity of IC clams was significantly higher in gills than in mantle, whereas in GB *A. islandica* no tissue specific differences were found (Table 2).

Table 2: Activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in U g^{-1}wetmass in gill and mantle of non-burrowed (*B-N*) or 3.5-days-burrowed (*B-MRD*) German Bight (GB) and Iceland (IC) *Arctica islandica*, and of GB clams with changing PO_2 in the mantle cavity water (*PO_2-N*) or of ≥ 24 h 0 % O_2 in the mantle cavity water (*PO_2-MRD*). Data in mean±s.d, each group *n* = 6-11, experimental temperature = 10 °C, assay temperature = 20 °C. # Significant differences in CAT of IC N clams between gill and mantle (Kruskal-Wallis $P < 0.0001$, Dunn´s $P < 0.05$).

	GB *B-N*	GB *B-MRD*	IC *B-N*	IC *B-MRD*	GB *PO_2-N*	GB *PO_2-MRD*
MRD duration	-	3.5 days	-	3.5 days	-	24 h
Gill SOD	704.6 ± 323.6	728.6 ± 226.1	632.0 ± 281.3	661.4 ± 149.1	731.8 ± 83.2	744.5± 185.3
Mantle SOD	545.4 ± 265.3	790.0 ± 430.6	607.5 ± 290.4	726.8 ± 420.1	767.2 ± 276.6	674.0 ± 203.6
Gill CAT	2944.4 ± 1016.1	4035.9 ± 1472.1	3907.8 ± 958.7#	4240.2 ± 1036.0	2464.5 ± 817.9	2769.0 ± 769.5
Mantle CAT	3361.8 ± 1114.2	2997.9 ± 1098.1	2757.5 ± 565.3	2903.6 ± 862.8	2751.8 ± 692.9	2804.4 ± 431.4

The overall adenylate concentration (ATP + ADP + AMP) was significantly higher in gills of GB *PO_2-N* clams compared to mantle, which was due to higher ATP and ADP concentrations (Table 3). AMP concentrations as well as energy charge (EC) and AMP/ATP ratio were similarly high in both tissues of *A. islandica*. Gill and mantle differed in their response to MRD condition. In the mantle total adenylate concentrations were significantly higher in *PO_2-MRD* clams compared to *PO_2-N* clams, mainly due to higher ADP and AMP concentrations after 24 h of 0 % O_2 in mantle cavity water. In contrast the gill had similarly high contents of total adenylates, especially of ADP and AMP but significantly lower ATP-concentrations under *PO_2-MRD* compared to *PO_2-N* conditions. Accordingly, EC was significantly lower in gills of *PO_2-MRD* than *PO_2-N* clams, but similar under both conditions in mantle. The AMP/ATP ratio was higher in mantle and gill in *PO_2-MRD* compared to *PO_2-N* clams, but differences were statistically not significant (Table 3).

Citrate synthase (CS) activity was similar in all tissues, but the ATP/CS ratio was significantly higher in gill compared to mantle in *PO_2-N A. islandica* (Table 3). Neither CS activity nor ATP/CS ratio were significantly altered by the mantle cavity PO_2 status (Table 3).

The succinate concentration in the adductor muscle of both GB and IC *A. islandica* was significantly higher in (buried) *B-MRD* clams compared to (non-buried) *B-N clams*, but of insignificant difference between *PO_2-N* and *PO_2-MRD* of the GB (Table 4). Succinate accumulation was significantly higher in 3.5-days burrowed IC than GB clams (Table 4).

Table 3: Adenylate concentrations in nmol g^{-1} WM and citrate synthase activity (CS) in U g^{-1}wetmass in gill and mantle of German Bight *Arctica islandica* with changing PO_2 in the mantle cavity water (*PO_2-N*) or ≥ 24 h 0 % O_2 in the mantle cavity water (*PO_2-MRD*). Data in mean±s.d., *n* = 9, experimental temperature = 10 °C, assay temperature of CS measurements = 20 °C, adenylate$_T$ =ATP + ADP + AMP. * and ** Significant differences in gill and mantle between *PO_2-N* and *PO_2-MRD* clams (One-way ANOVA P < 0.05, Tukey P < 0.05). ## Significant differences between gill and mantle (Two-way ANOVA P < 0.001), - no significant differences.

	gill *PO_2-N*	gill *PO_2-MRD*	mantle *PO_2-N*	mantle *PO_2-MRD*	Status	Tissue
Adenylate$_T$	352.1 ± 67.4 ##	292.1 ± 71.1	191.2 ± 55.7	290.3 ± 82.1 *	* P < 0.05	## P < 0.001
ATP	214.0 ± 42.4 ##	134.2 ± 39.3 **	109.2 ± 41.7	119.4 ± 34.9	** P < 0.001	## P < 0.001
ADP	86.8 ± 27.6 ##	83.8 ± 23.2	37.2 ± 11.7	88.1 ± 31.6 **	** P < 0.001	## P < 0.001
AMP	51.9 ± 24.5	74.0 ± 33.4	44.8 ± 25.3	82.8 ± 28.3 *	* P < 0.05	-
EC	0.73 ± 0.08	0.61 ± 0.09*	0.67 ± 0.12	0.57 ± 0.07	* P < 0.05	-
AMP/ATP	0.26 ± 0.17	0.60 ± 0.36	0.47 ± 0.39	0.73 ± 0.30	-	-
CS	1.75 ± 0.68	1.81 ± 0.80	2.47 ± 0.59	2.40 ± 0.67	-	-
ATP/CS	142.0 ± 64.1 ##	86.6 ± 34.5	48.1 ± 25.8	56.3 ± 34.2	-	## P < 0.001

Table 4: Succinate content in nmol mg^{-1}WM in the adductor muscle of non-burrowed (*B-N*) or 3.5-days-burrowed (*B-MRD*) German Bight (GB) and Iceland (IC) *Arctica islandica*, and of GB clams with changing PO_2 in the mantle cavity water (*PO_2-N*) or of ≥ 24 h 0 % O_2 in the mantle cavity water (*PO_2-MRD*). Data in mean±s.d., each group *n* = 7-10, experimental temperature = 10 °C, - no MRD. ** Significant differences in the GB and IC population between N and MRD clams (One-way ANOVA P < 0.0001, Tukey P < 0.001). # Significant differences between GB MRD and IC MRD clams (One-way ANOVA P < 0.0001, Tukey P < 0.01).

	Succinate	Duration of MRD
GB *B-N*	5.65 ± 1.44	-
GB *B-MRD*	65.49 ± 19.07 **	3.5 days
IC *B-N*	7.92 ± 3.13	-
IC *B-MRD*	90.71 ± 25.34 ** #	3.5 days
GB *PO_2-N*	5.69 ± 3.11	-
GB *PO_2-MRD*	10.09 ± 4.24	24 h

Discussion

Burrowing behaviour in *A. islandica* greatly varied with season and likely depends on seasonal feeding conditions and temperature. In June, when phytoplankton concentrations at the study site in Eyjafjördur North Iceland are 30-times higher than in February (Kaasa and Guðmundsson 1994) 50 % of all bivalves burrowed directly beneath the sediment surface. Siphons of shallow burying bivalves are in direct contact with the overlying sea water to take up oxygen and food. Predation has been suggested to be a major burrowing elicitor in bivalves (Griffith and Richardson 2006), but the massive shells of adult *A. islandica* apparently provide sufficient protection from predators such as fish (Arntz and Weber 1970). Thus, low food conditions in winter appear to be one of the major extrinsic causes of greater seasonal burrowing depth in *A. islandica*, and further, low water temperatures also support metabolic arrest in bivalves (Morley et al. 2007). In the inter-population comparison IC clams may burrow more frequently and for longer periods than GB *A. islandica* because of a shorter annual feeding period. The spring phytoplankton blooms around North Iceland last only 6 weeks from early April to mid May followed by much lower summer and autumn values (Gudmundsson 1998). Contrary, bloom season at Helgoland is from April to the end of August (http://www.bsh.de/de/Meeresdaten/Beobachtungen/MURSYS-Umweltreportsystem/Mursys_031/seiten/noph2_01.jsp).

MRD in *A. islandica* is a voluntary behavior, which is impressively illustrated by the mantle cavity PO_2 measurements and the ANCOVA model of PO_2 residuals over time in spontaneously 'hibernating' bivalves. Interestingly, depression of metabolism in the ocean quahog is not exclusively linked to, or a consequence of, burrowing into deeper sediment horizons, but it is also self-induced when the bivalves are deprived of their sedimentary retreat and, instead, maintained in normoxic sea water (see also Abele et al. 2010). More than 30 % of GB and IC clams did not respire for > 24 h during the measurements of whole animal respiration (see Table 1). An intrinsic and species specific burrowing pattern, independent of water temperature and feeding, was visible in both populations during laboratory experiments and, according to investigations of an Irish Sea population (Taylor 1976), individual periods of constant burrowing lasted between 1 and 6 days. In preparation for their short term 'hibernation', *A. islandica* show a distinct breathing behaviour several hours before they enter the metabolically reduced status. Enhanced oxygen uptake may increase ATP production just before closing the shell or burrowing down, presumably to meet energy requirements which may be not yet down-regulated during the first hours of shell-closure, or during burrowing exercise. Bivalves which merely closed their shells for 24 h accumulated no succinate, whereas *A. islandica* that burrowed into the sediment for longer periods of 3.5 days had increased succinate levels in the adductor muscle.

Arctica islandica are well adapted to hypoxia and maintain low internal oxygenation also under normoxic environmental conditions. Mean mantle cavity PO_2 in GB specimens was < 5 kPa during siphon-opening and -closure, and the same was found in a parallel experimental study with Kiel

Bight *A. islandica* (Abele et al. 2010). Steady control of mantle cavity oxygenation may be instrumental in achieving low and protective PO_2 levels in cells of hypoxia tolerant and oxygen sensitive animals (Massabuau 2003). However, *A. islandica* can be distinguished from other bivalves such as *Mya arenaria* through the specific 'rhythms' of mantle cavity PO_2 oscillations. Whereas the deep burrowing soft shell clam *M. arenaria* maintains mantle cavity PO_2 nearly constant between 0 and 2.6 kPa (Abele et al. 2010), GB *A. islandica* in the present study oscillated PO_2 between 2 kPa and 21 kPa with a period of 50 min. Kiel Bight animals measured by Abele et al. (2010) oscillated in the same PO_2 range but more slowly with periods of 96 and 250 min. Thus, there may be population specific patterns of shell water ventilation in *A. islandica*: with more frequent ventilation in bigger North Sea specimens and less frequent ventilation in smaller Baltic Sea specimens. However, both ventilation patterns lead to the adjustment of the same mean PO_2 level in mantle cavity water, which seems to be species specific and adaptive for *A. islandicas´* shallow and frequently burrowing lifestyle. Indeed, mean PO_2 of the ocean quahog is not as low as in the deep burrowing (± 50 cm) soft shell clam *M. arenaria* and not as high as in epibenthic swimming scallops (Abele et al. 2010).

The mitochondrial capacity, represented by CS activity and the ATP/CS ratio, remained constant in mantle and gill of GB bivalves between normoxia and the first 24 h of MRD. Nevertheless, ATP utilization in the gills seems to outrun ATP production, possibly due to continued ventilatory activity of the cilia when the shells are closed, and anaerobic energy production has not yet started. Thus ATP content and EC decreased and succinate, which is the first intermediate signaling the onset of anaerobic metabolism (Oeschger 1990; Strahl et al. 2011), remained constantly low during ≥ 24 h of 0 % O_2 in the mantle cavity water of *A. islandica*. The gills of GB *A. islandica* are metabolically much more active than the mantle. Higher overall adenylate content and higher ATP/CS ratio, indicating higher phosphorylation efficiency, and further higher SOD and CAT activities were detected in gill compared to the mantle tissue. Correspondingly, tissue respiration (Strahl et al. 2011), and even cell-turnover rates are higher in gills compared to mantle of *A. islandica* (Tschischka et al. 2000; Strahl and Abele 2010). Maintenance of the energy charge and higher ATP concentrations in the generally less active mantle tissue of *A. islandica* after >24 h of 0 % O_2 in the mantle cavity water can be attributed to a slow-down of metabolism. This was also found in 6h air exposed limpets, *Patella vulgata*, which maintained EC and increased ATP content in the foot muscle compared to control conditions without inducing anaerobiosis (Brinkhoff et al. 1983). The AMP/ATP ratio in MRD bivalves increased in both gill and mantle, which at a certain threshold activates glycolysis, lipidoxidation and anaerobic metabolism via the AMP-activated protein kinase (AMPK) pathway (Taylor 2008). AMPK down-regulates energy consuming anabolic processes and supports hypoxic survival in a metabolically reduced status.

A life prolonging effect of intermittent MRD may be based on the transient reduction of metabolically derived ROS formation (Abele et al. 2010, Buttemer et al. 2010). Although *A. islandica* already features very low *in-vitro* ROS formation under normoxic state 3 and 4 (Buttemer et al. 2010), ROS

production in isolated gill tissue was found to be drastically reduced under low oxygen conditions of 5 kPa compared to 21 kPa and supposedly fully subsides as cellular respiration stops at 0 kPa. The critical point is that oxidative burst may be happening when the bivalves surface, and cells are flooded with oxygen. Our data on enzymatic antioxidants in this paper, as well as on glutathione levels during hypoxic exposure of *A. islandica* in a previous paper (Strahl et al. 2011) indicate no anticipatory antioxidant response takes place. In agreement a ROS-burst was absent in isolated gill tissue of IC *A. islandica* (present study) and of GB clams (S. Hardenberg unpublished) after hypoxia-reoxygenation and ROS-levels were much lower than under constant normoxic exposure. An alternative oxidase pathway in mitochondria of *A. islandica* during reoxygenation may act as respiratory protection by increasing the rate of cellular oxygen consumption, and thereby lowering the tissue PO_2 and minimizing the risk of oxyradical formation (Tschichka et al. 2000). Thus there is little need to protect tissues in surfacing clams. The extremely high superoxide-level in gills immediately after dissection seems to be an artifact showing ROS-formation as a stress response in freshly excised samples.

In conclusion, *A. islandica* spontaneously induce a metabolically reduced and energy saving status at all times of the year, but this behaviour seems to be distinctive during winter, when food availability and water temperature are low. Biogeographic acclimation seems not decisive for this behaviour, and North Sea bivalves burrow at similar rates as bivalves from North Iceland when maintained under the same conditions. We conjecture that external factors such as climate change induced warming and intensive bottom trawling in the North Sea reduce *A. islandica* lifespan in the GB (see Strahl and Abele 2010). Further shorter annual feeding periods and colder annual temperatures in Icelandic waters may lead to longer burrowing-periods, and thus prolong individual MLSP in the IC population. Changes of adenylate concentrations, especially in the AMP/ATP ratio seem instrumental in regulating metabolism under MRD. Avoiding accumulation of anaerobic metabolites in the burrowed status limits the need for enhanced recovery respiration during surfacing and therewith avoids an oxidative burst reaction during re-oxygenation. Consequently, neither the levels of enzymatic nor low molecular antioxidants such as glutathione are enhanced in preparation for an oxidative burst, at least on the protein and activity level. Investigation on the expression level (mRNA) of antioxidant genes could give further information to what extent this species is prepared for re-oxygenation.

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4. CONTRIBUTED WORK

List of publications and declaration of my contribution towards them:

Publication IV

Begum S, Basova L, Strahl J, Sukhotin A, Heilmayer O, Philipp E, Brey T, Abele D (2009) A metabolic model for the ocean quahog *Arctica islandica* - effects of animal mass and age, temperature, salinity, and geography on respiration rate. Journal of Shellfish Research 28: 533-539

I sampled animals from one *A. islandica* population in the German Bight out of overall five geographically separated populations in the North East Atlantic. I provided all data of the German Bight *A. islandica*. The manuscript was written by S. Begum and improved in cooperation with all co-authors.

Publication V

Basova L, Begum S, Strahl J, Sukhotin A, Brey T, Philipp EER, Abele D (in revision) Age dependent patterns of antioxidants in *Arctica islandica* from six regionally separate populations with different life spans. Aquatic Biology

I sampled animals from two *A. islandica* populations in the German Bight and around Iceland out of overall six geographically separated populations in the North East Atlantic. I provided all data of the Iceland *A. islandica* and part of the German Bight *A. islandica* data. The manuscript was written by L. Basova and revised by all co-authors.

Publication VI (= Book section)

Philipp EER, Strahl J, Sukhotin AA. Aging in marine animals. In: Abele D, Vázquez-Medina JP, Zenteno-Savín T (in press) Oxidative Stress in Marine Ecosystems. Wiley-Blackwell. UK. ISBN: 9781444335484.

I wrote the paragraph ‘Technical section: age estimation in marine organisms’ (including: *Age estimation using hard structures, Biochemical and physiological proxies of aging*), which is a part of the book section ‘Aging in marine animals’. The complete book section was improved in cooperation with all co-authors and D. Abele (Editor of the book).

PUBLICATION IV

A metabolic model for the ocean quahog *Arctica islandica* – Effects of animal mass and age, temperature, salinity, and geography on respiration rate

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Abstract

Owing to its extraordinary lifespan and wide geographical distribution along the continental margins of the North Atlantic Ocean, the ocean quahog *Arctica islandica* may become an important indicator species in environmental change research. To test for applicability and 'calibrate' the *A. islandica*-indicator, metabolic properties of *A. islandica* specimens were compared across different climatic and oceanographic regions. Fully saline populations from Iceland to the North Sea as well as animals from polyhaline and low salinity, environments, the White Sea and the Baltic Sea were included in the study. This calibration centrally includes recordings of growth-age relationships in different populations. Shells were used as age recorders by counting annual growth bands. As a result of this study, we propose a general respiration model that links individual metabolic rates of *A. islandica* from five populations: Norwegian coast, Kattegat, Kiel Bay (Baltic Sea), White Sea and German Bight (North Sea), to body mass, water temperature and site. Temperature exerts distinct site specific effects on respiration rate, which is indicated by Q10 values ranging from 4.48 for German Bight to 1.15 for Kiel Bay animals. Individual age, occurrence of apneal respiratory gaps, parasite infestation and salinity do not affect respiration rate. Respiration of *A. islandica* is significantly below the average of 58 bivalve species when compared at the same temperature and animal mass. This respiration model principally enables the coupling of *A. islandica* life history and population dynamics to regional oceanographic temperature models.

Keywords

Arctica islandica, age, mass, respiration, site, temperature

Introduction

The ocean quahog *Arctica islandica* inhabits the continental shelves and slopes at depths between 4–482 m on both sides of the North Atlantic Ocean along a latitudinal range to the north from Cape Hatteras (~35°N), on the Western Atlantic coast and from the Wadden Sea near Texel (~54°N), to the Barents Sea (~70°N) on the East Atlantic coast (Nicol 1951, Thompson et al. 1980a, Thompson et al. 1980b, Murawski et al. 1982, Jones 1980, Dahlgren et al. 2000). *A. islandica* tolerates seawater temperatures between 0°C and 16°C and has an optimal range between 6°C and 10°C (Mann 1982). The maximum recorded age of the ocean quahog is close to 400 years, and individuals over 100 years are abundant in the North Atlantic (Schöne et al. 2005b, Strahl et al. 2007). Its extreme longevity and wide geographical distribution makes *A. islandica* an attractive model for studies of recent and past climate change (Jones 1980, Thompson et al. 1980a, Murawski et al. 1982, Schöne et al. 2005b) as the shells archive information on environmental conditions that individual animals experience over lifetime (Schöne 2003, Schöne et al. 2004; Witbaard et al. 2003; Epplé et al. 2006, Schöne et al. 2005b).

To read and interpret the *A. islandica* shell archive, we need a sound knowledge of the interacting effects of extrinsic (environmental) and intrinsic (physiological and genetic) factors, which may modify the ageing process in this bivalve species (Abele et al. 2008). The mass specific standard respiration rate (MSR) is a measure of the intensity of basal aerobic metabolism and is primarily controlled by the habitat temperature in ectotherms. MSR has been related to the rate of physiological aging, because higher lifetime oxygen consumption per unit tissue mass accelerates senescent processes in cells and tissues (Pearl 1928, Harman 1956, Sohal 1986, Philipp et al. 2005a). This, in turn, means that cold adapted mollusks of any given species can be expected to have longer lifespan than their congeners from warmer waters, as documented for the pearl shell clam *Margaritifera margaritifera* (Ziuganov et al. 2000).

Arctica islandica are known to modulate their metabolism by performing metabolic shut-downs at irregular intervals in which metabolism is rapidly reduced to as low as 10% of the standard metabolic rates. These metabolically depressed states referred to as metabolic rate depression (MRD), in which the animals burrow a couple of centimeters into the sediment for 1 to 7 days (Taylor 1976, Thompson 1984) and which may represent a life prolonging mechanism in *A. islandica* (Abele 2002, Strahl et al. 2007). In addition, active non-burrowing animals exhibit respiratory breaks. Another behavioral characteristic of *A. islandica* are shorter lasting apneal phases (APs), which do not necessarily involve reduction of the metabolic rate (MRD). These transient respiration breaks (i.e., ventilation stops) which last for no more than a couple of minutes, have already been described for other bivalve species (Morley et al. 2007).

Measurements of oxygen consumption rates (e.g. MSR) over age allow to model individual lifetime energy expenditure and serve as the approximation of population specific energy budgets (Brey

2001). MSR correlates with mass and age in bivalve mollusks and normally decreases in larger specimens (e.g. Robertson 1979, Ikeda 1985, Hawkins and Bayne 1992, Heilmayer and Brey 2003, Heilmayer et al. 2004, Clarke and Fraser 2004).

Here, we determined MSR of *A. islandica* from five populations (Kattegat, Kiel Bay, German Bight, Norwegian Coast, and White Sea) at mean site specific habitat temperature (HT) and moreover upon acclimation to 5°C above HT (elevated temperature = ET). Experimental warming within the natural temperature window allows to study the effect of temperature on respiration rate in a population and to compare the response to warming between populations of distinct climatic background. Further, we analyzed the frequency and duration of the apneal respiratory pauses (AP) and their dependence on environmental factors (= temperature, salinity) and on individual age and size. Based on these measurements, we developed a model of *A. islandica* respiration from the North Atlantic populations, which allows to answer the following questions:

(i) How do environmental parameters such as salinity, food, and parasites influence respiration of *A. islandica*?
(ii) How does seawater temperature influence respiration of *A. islandica* with different climatic background?
(iii) How is respiration related to mass and age in each population?
(iv) Do AP phases affect lifetime respiration in different populations?

Materials and Methods

Sampling Sites and Maintenance

In 2006 *A. islandica* were collected from five different geographic locations (Fig. 1) covering a temperature and salinity gradient of 4–10°C and 25–34 PSU, respectively (Table 1). Bivalves were transported alive to the Alfred Wegener Institute (AWI, Germany) and were kept for 4–6 weeks at mean annual habitat temperature of the respective sampling site prior to experimentation (Table 1). Animals were maintained in 60-L flow-through aquaria containing 8 cm of sediment and natural seawater of site-specific salinity. The bivalves were fed once a week with DT-live marine plankton (DT, USA, 1 mL^{-1} $animal^{-1}$ $week^{-1}$).

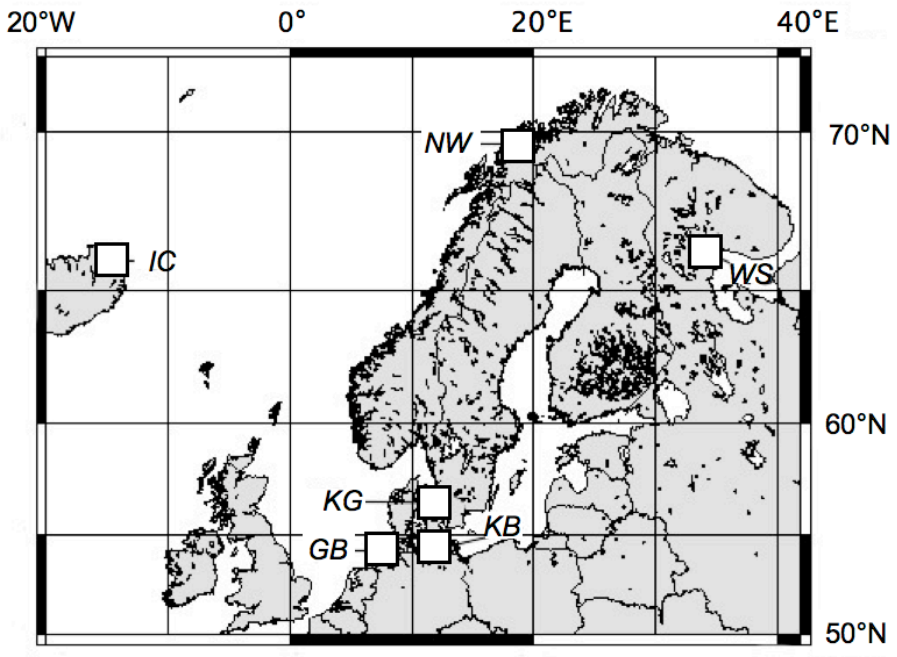

Fig 1: Sampling locations of *Arctica islandica* (white squares), NW: Norwegian coast, KG: Kattegat, GB: German Bight, WS: White Sea and KB: Kiel Bay. Map generated by online map creator at www.aquarius.ifm-geomar.de

Table 1: Geographical area of *Arctica islandica* collection (Location), environmental salinity at locations (Salinity), sampling depth (Depth), experimental temperature (HT: mean annual habitat temperature; ET: elevated temperature) and sample size for each *A. islandica* population.

Population	Location	Salinity (PSU)	Depth (m)	Temperature (°C)		Sample size (n)	
				HT	ET	HT	ET
Norwegian Coast	69°39´N 18°57´E	33	10-30	4	9	35	23
Kattegat	56°10´N 11°48´E	31	33	8	13	29	16
White Sea	66°18´N 33°38´E	25	10	4	9	12	12
Kiel Bay	54°32´N 10°42´ E	25	25	10	15	28	24
German Bight	54°09´N 07°47´E	31	40	10	15	8	10

Experimental Setup

Animals of each population were randomly assigned to two different groups. The first subsample was maintained at site-specific mean annual habitat temperature (HT), whereas the second subsample was acclimated stepwise (1°C temperature increase every 2 days) to 5°C above mean habitat temperature (elevated temperature = ET) (see Table 1). ET bivalves were kept for at least four weeks at the elevated temperature prior to respiration measurements.

Measurement of Respiration

Respiration was measured in a multichannel modified intermittent flow system as described by Heilmayer and Brey (2003). Prior to measurements, *A. islandica* were maintained without food for three days, to eliminate the impact of specific dynamic action (SDA) on respiration (Bayne et al. 1976). Bivalves were allowed to accommodate to the respiration chambers overnight, and oxygen consumption was recorded only in actively respiring animals that had their siphons open. Respiration chambers were Perspex cylinders that allowed adjusting chamber volume between 100–600 ml to animal size (Heilmayer and Brey 2003). Experimental temperature was maintained stable (±0.5°C) by placing the chambers in a water bath within a water-jacketed container, thermostatted using a thermo circulator (Julabo FP 40, Germany). Three respiration chambers, each with one animal and a control chamber without animal were used for simultaneous measurement in each experiment. After each measurement, the animal was carefully removed from the chamber and oxygen consumption recorded for another 3 h to determine the microbial oxygen demand in the respective chamber. Oxygen content in the chambers was monitored continuously with oxygen microoptodes connected to a MICROX TX3 array (PreSens, Neuweiler, Germany). Optodes were calibrated to 100% oxygen solubility in air-saturated and to 0% in N_2-saturated seawater (technical gas with 99.996% N_2) at each experimental temperature. Immediately after the measurements, animals were dissected, and examined for the presence of the parasitic Nemertean worm, *Malacobdella grossa*. Soft tissue was dried at 68°C for at least 48 h to obtain dry mass (DM). Dried tissues were combusted 24 h at 500°C to calculate ash free dry mass (AFDM = DM – ash). Individual age was inferred from shell growth bands (see below).

Calculation of Metabolic Rates

Oxygen consumption rates (VO_2, mmol O_2 h^{-1} ind^{-1}) were determined from the slope of the oxygen saturation curve after subtraction of the microbial oxygen demand. Percent O_2 saturation was transformed to O_2 volume concentration using known values of oxygen solubility (Benson and Krause 1984).

$$VO_2 = \frac{sat\, t_0}{sat\, t_{60}} \cdot \alpha_{O_2} \cdot V_{Chamber} \qquad (1)$$

VO_2: volume of oxygen consumed (mmol O_2 h^{-1} ind^{-1}), αO_2 : oxygen solubility in sea water (mmol dm^{-3}), $V_{Chamber}$: volume of respiration chamber and tubing (dm^3), sat t_0: oxygen saturation (%) at the beginning of the experiment and sat t_{60}: oxygen saturation (%) after 60 min of respiration

in the closed system. Apneal respiration (AP) events, manifesting as time periods (intervals) with zero oxygen consumption (Fig. 2), were recorded (frequency and duration) and are included in the computation of overall respiration rate.

Individual mass-specific respiration rates (MSR, mmol O_2 h^{-1} g $AFDM^{-1}$) were calculated according to:

$$MSR = \frac{VO_2}{AFDM} \quad (2)$$

The temperature coefficient Q10 was calculated from the overall multiple linear model for mass specific respiration rates between two different temperatures (5°C and 10°C), as

$$Q_{10} = \left(\frac{MSR_2}{MSR_1}\right)^{\frac{10}{t_2 - t_1}} \quad (3)$$

where MSR is the mass specific respiration and t is temperature.

Individual Age Determination

For the age analysis left shell valves were cleaned with warm NaOCl (5%) solution, rinsed with demineralized water and dried at 60°C for 12 h. Each valve was embedded in epoxy resin (Wiko liquid metal FLM-S25), sectioned along the axis of strongest shell growth and dried overnight. Big valves (≥50 mm) were cut with a table diamond saw (FK/E PROXXON-28070, Germany). Smaller valves (<50mm) were mounted on a Plexiglas block for easier handling during the preparation process and cut with a Buehler low-speed diamond saw (Isomet 1000™, USA). Cross-sections were ground on a Buehler low and high speed Grinder and Polisher, using grits of P400, P1200, P2400, and P4000 grade and subsequently polished using a polycrystalline diamond suspension of 1 and 0.1 mm (Buehler, USA). The polished shell section was immersed in Mutvei's solution for 20 min at 37°C, following the protocol of Schöne et al. (2005a). Immediately afterwards, the etched section was rinsed with demineralized water and allowed to dry on air, resulting in a very clear, three-dimensional growth pattern that reveals distinct annual growth lines. Growth increments were analyzed under a reflected-light stereomicroscope (Olympus SZX12, USA) and digitalized with an Olympus camera (Olympus U-CMD3 Colorview, USA) at 10°–90° angle. Annual growth increments in the outer shell layer were counted following Schöne et al. (2005b) using the image analysis software 'analySIS 5.0' (Olympus Soft Imaging Solutions, USA).

Statistical Analysis

We used analysis of variance (ANOVA) and analysis of covariance (ANCOVA) to explore the relationships between mass-specific respiration rate ln(MSR), apneal respiration behavior (AP), body mass ln(M), age, temperature 1/T (Kelvin), salinity and site (geographical area of collection).

Owing to the strong correlation between body mass and age, these two parameters could not be analyzed simultaneously. We tested for the effects of age on MSR in two different ways: by correlating the residuals of the final multiple linear model with age, and by testing for age effects in a data subset that covered a small body mass range but a wide age range. Effects of temperature, salinity, body mass and age on frequency and duration of apneal phases were analyzed by full interaction ANCOVA. Mahalanobis distances (Barnett and Lewis 1994) were used to identify multivariate outliers that were excluded from further analysis. All analyses were carried out using the statistical package JMP by SAS Inc (1988, USA).

Results

Table 2 summarizes the basic information on the 197 quahogs from the five populations we sampled. The number of data available for statistical analysis reduced to 193 after Mahalanobis analysis identified 4 outliers.

The German Bight population was outstanding because of the lack of small/young animals below 3.7 g and 33 years, no detectable nemertean infestation (n = 18), and because of the highest (albeit not significantly different) recorded percentage of apneal phases (AP) (80%, Table 2).

Table 2: Total number of measured *Arctica islandica* in the respiration experiments (n) at different experimental temperatures, *M. grossa*: frequency of occurrence of infestation with Nemertean *Malacobdella grossa,* AP: frequency of occurrence of apneal respiration events (periods of reduced respiration). nd: not determined

Population	Temperature (°C)	Sample size (n)	Mass Range (g AFDM)	Age range (years)	*M. grossa* *(%)*	AP (%)
Norwegian Coast	4	35	0.35 – 12.01	6 – 93	68.57	34.29
	9	23	0.04 – 12.01	4 – 90	56.52	30.43
Kattegat	8	29	1.11 – 2.95	8 – 71	43.75	38.00
	13	16	0.91 – 2.95	11 – 45	41.38	37.50
White Sea	4	12	0.03 – 0.38	3 – 31	41.67	33.33
	9	12	0.12 – 0.42	12 – 53	50.0	41.67
Kiel Bay	10	28	0.06 – 1.87	4 – 29	10.71	28.57
	15	24	0.08 – 1.42	nd	8.33	33.33
German Bight	10	8	5.04 –7.34	33 –98	0.00	87.50
	15	10	3.71 – 6.96	38 –94	0.00	80.00

During an AP event the animals stopped or considerably slowed respiration for between 3 and 31 min and subsequently continued respiration at rates similar to pre-AP respiration (Fig. 2). This apneal respiration behavior was observed in about one third of all measurements in all five populations. In those animals that showed APs, average frequency and duration was 0.50 ± 0.88

AP events h^{-1} and 5.0 ± 8.0 AP min h^{-1}. Neither temperature, nor salinity, body mass, or age affected AP frequency or duration in a significant manner (full interaction ANCOVA).

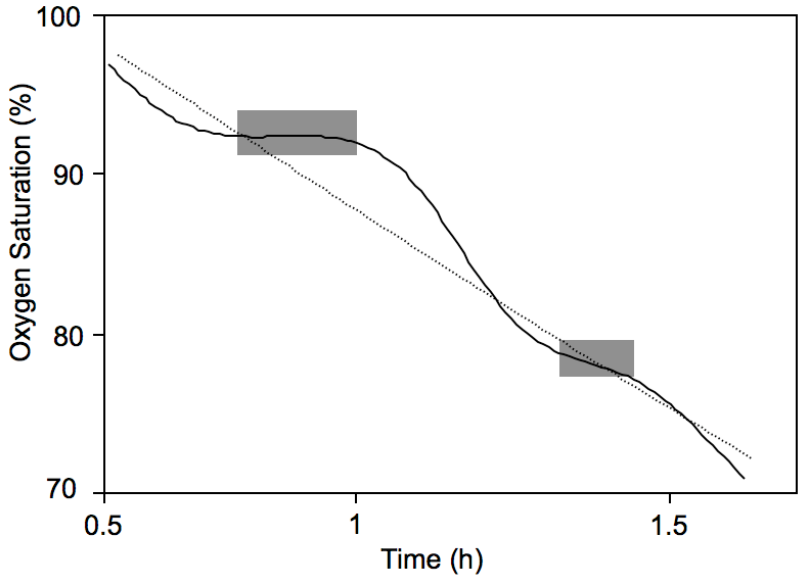

Fig 2: Typical apneal respiration event pattern (AP) during the course of the respiration measurement of one *Arctica islandica* specimen (Norwegian Coast, 9°C). Grey boxes indicate AP periods characterized by distinctly slower decrease in oxygen saturation. Dotted line shows oxygen decline in a similarly sized individual with no AP.

Neither the salinity regime (euhaline versus polyhaline) nor nemertean infestation (yes/no) significantly affected mass specific respiration. MSR was significantly affected by body mass (AFDM), temperature, site and the interactions between these parameters, whereas AP behavior, parasite infestation, and salinity showed no effect. The relationship is described best by the full factorial multiple linear model,

$$\ln(MSR) = 22.156 - 0.224 * \ln(M) - 5831.651/T + b_{3,SITE} + b_{4,SITE} * \ln(M) + b_{5,SITE}/T$$

n = 193, R^2 = 0.656, P < 0.001; [mmol O_2 h^{-1} g^{-1} AFDM, g AFDM, Kelvin] where $b_{3,SITE}$; $b_{4,SITE}$; and $b_{5,SITE}$ are SITE specific parameters (see Table 3 for parameter values). When SITE effects are not considered, the model reduces to

$$\ln(MSR) = 17.592 - 0.203 * \ln(M) - 4452.9171/T$$

n = 193, R^2 = 0.493, P < 0.001; [mmol O_2 h^{-1} g^{-1} AFDM, g AFDM, Kelvin].
Negative age effects on MSR (= older animals respiring less) were not detected. The residuals of the multiple linear model were not correlated with age (P = 0.11). Within the data subset ranging from 6–12 g AFDM and from 41–94 years of age, MSR was independent of body mass (P = 0.825, model: ln(MSR) versus ln(M), 1/T and SITE), allowing for an independent test of the effect of age

on respiration. The corresponding model that uses age instead of ln(M) also indicates no significant effect of age on MSR (P = 0.158).

Figure 3 visualizes the effects of body mass (negative) and temperature (positive) on MSR. The residuals of the full factorial model are distributed randomly (Fig. 4); our model is an accurate descriptor of the relationship between dependent and independent parameters. Based on the model, Q10 values (5°C–15°C) were 4.48 for German Bight, 2.63 for Norwegian Coast, 2.34 for White Sea, 1.20 for Kattegat and 1.15 for Kiel Bay.

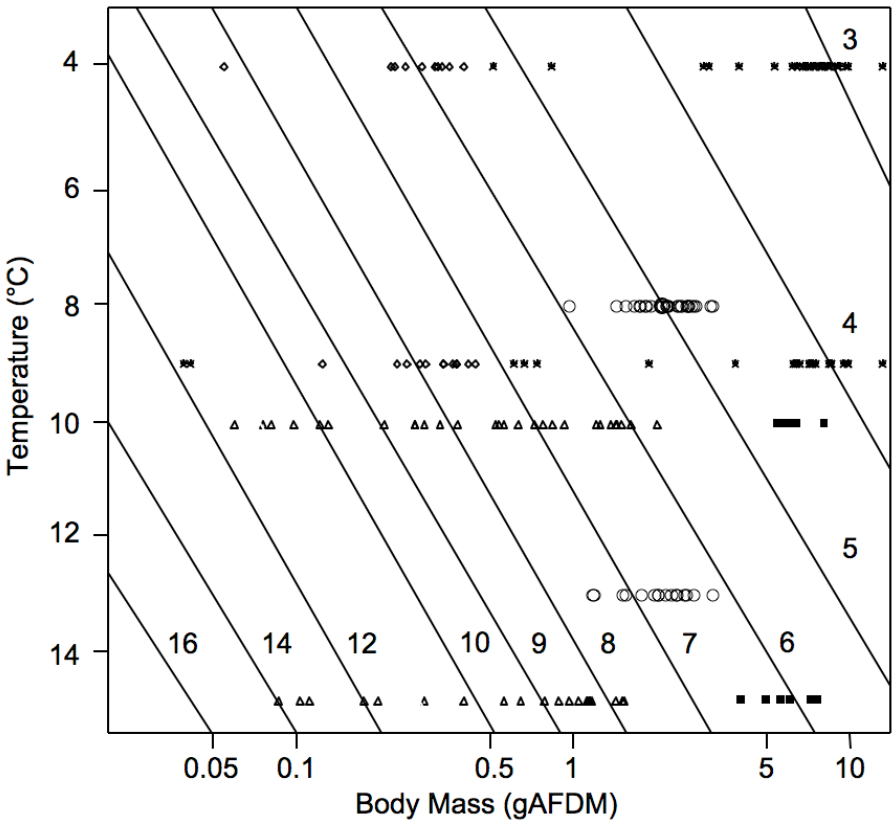

Fig 3: Visualization of the relationship between MSR (µmol O_2 h^{-1} g^{-1} AFDM) of *Arctica islandica* from different locations, body mass (g AFDM) and temperature (here shown in °C) as described by the reduced multiple linear model (SITE effects neglected, Table 3). Lines represent MSR isopleths ranging from 2 to 14 µmol O_2 h^{-1} g^{-1} AFDM. Superimposed are the body mass / temperature data of all MSR measurements. Triangles: Kiel Bay, diamonds: White Sea, squares: German Bight, stars: Norwegian Coast, circles: Kattegat.

Table 3: Parameter values of the multiple linear prediction model. $\ln(MSR) = 22.156 - 0.224 * \ln(M) - 5831.651 / T + b_{3,SITE} + b_{4,SITE} * \ln(M) + b_{5,SITE} / T$; [µmol O_2 h^{-1} g^{-1}; g AFDM; Kelvin]; n = 193, R^2 = 0.656, $p < 0.001$. Reduced model (SITE effects neglected): $\ln(MSR) = 17.592 - 0.203 * \ln(M) – 4452.917 / T$; n = 193, R^2 = 0.493, $p < 0.001$

Site	$b_{3,Site}$	$b_{4,Site}$	$b_{5,Site}$
Norwegian Coast	7091	0.144	-1923777
Kattegat	-14984	0.176	4339745
White Sea	2947	-0.483	-967735
Kiel Bay	-16272	-0.026	4732580
German Bight	21218	0.190	-6180815

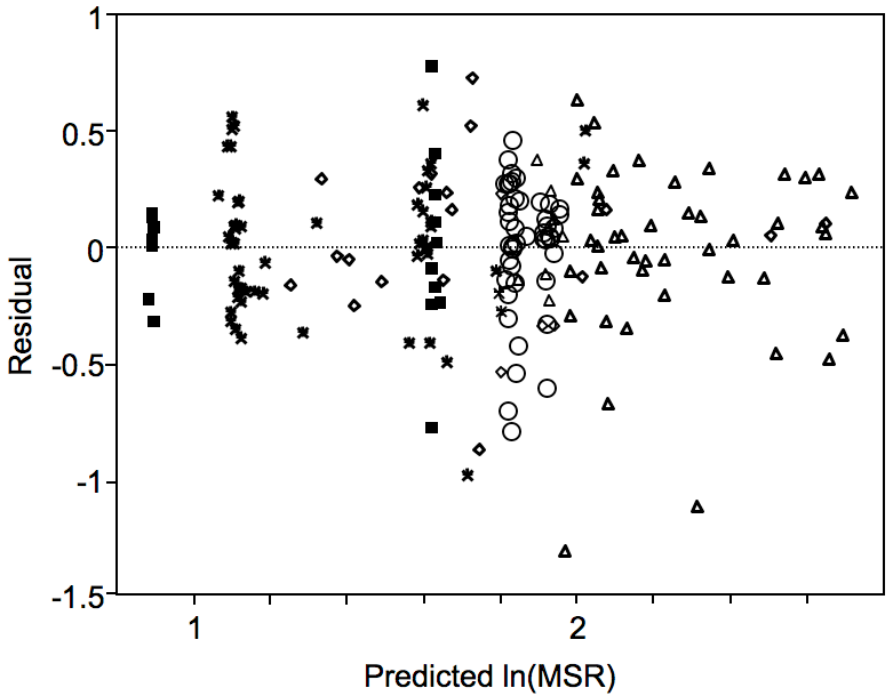

Fig 4: Residual plot (residual versus predicted values) of the full factorial multiple linear model (Table 3). Triangles: Kiel Bay, diamonds: White Sea, squares: German Bight, stars: Norwegian Coast, circles: Kattegat.

Discussion

The intention of this study was to build a general predictive model of respiration in *A. islandica*, taking into account significant biotic (body mass, age, parasites, apneal respiration behavior) and abiotic (temperature, salinity, SITE) parameters. The model (Table 3, Fig. 3) is of acceptable accuracy and precision (Fig. 4) and fits external data (as shown later). There are however two basic constraints that should be kept in mind when applying this model. Firstly, the body mass range differs greatly between populations, from small animals (≤0.42 g AFDM) in the White Sea group to mostly large bivalves (≥3.71 g AFDM) sampled in German Bight (Table 2). We tried to

minimize these differences through the selection of the most similarly sized experimental animals, but had to deal with the material available. This discrepancy may have biased SITE effects to an unknown extent, particularly for SITE-body mass and the SITE-temperature interactions. Secondly, besides temperature, salinity, water depth, and geographical location, we know little about the five SITEs. Hence, we are not able to explain the causes of the observed SITE effects. Possibly, stress (e.g. caused by hydrodynamics or by water sediment load; Jarmillo et al. 2008) or food availability (e.g. different levels of primary production; Sejr et al. 2004) may play a role. Therefore, we recommend to apply the reduced model (SITE effects neglected) to predict MSR of *A. islandica* from other populations and areas. This may reduce accuracy in the absolute values, but will maintain the precision of the predicted trends.

For a first testing, we used this reduced model to compare predicted values of MSR with those measured by Taylor and Brand (1975) in *A. islandica* collected from Laxey Bay, Isle of Man as well as off the Danish coast. The data set consists of 30 measurements carried out by Taylor and Brand (1975) and 11 additional data points taken from Bayne (1971). Respiration was measured at 10°C, and the study was aimed at investigating the effect of decreasing PO_2 on *A. islandica* (i.e. measurements continued down to 10% [2–3 kPa] of normoxic oxygen saturation [21 kPa = 100% PO_2]).

Below 5 mmol O_2 h^{-1} g^{-1} the MSR-model accuracy was rather good (<1 mmol), but decreased constantly with increasing MSR (i.e. measured MSR was increasingly underestimated at higher consumption values, Fig. 5). A matched pair test confirms this view, measured and predicted MSR do not differ significantly below 20 mmol O_2 h^{-1} g^{-1} of measured MSR ($P = 0.968$), but the difference becomes significant ($P < 0.001$) when the whole range of data is compared. Hence, the small animals ($n = 11$) with high MSR cause a problem (Fig. 5). Interestingly, all of these have been measured by Bayne (1971) so that we cannot exclude a consistent methodical bias. These small animals may have been in poor physiological condition and may have lost their ability to regulate oxygen consumption under hypoxia, as suggested by Taylor and Brand (1975).
Some unexpected findings arose from our measurements, which need to be given some further consideration:

Why is there no age effect in A. islandica metabolism?

The lack of negative age effects on respiration can be explained in the context of an apparent maintenance of physiological fitness and only minor accumulation of senescence indicators over age (i.e. fluorescent age pigments and protein carbonyl accumulation) in the extremely long-lived Icelandic *A. islandica*. In these long-lived animals, the activity of the mitochondrial marker enzyme citrate synthase (CS) remained constant in mature animals from 33 years into old age, with our oldest experimental specimens approaching 200 years (Strahl et al. 2007, Abele et al. 2008).

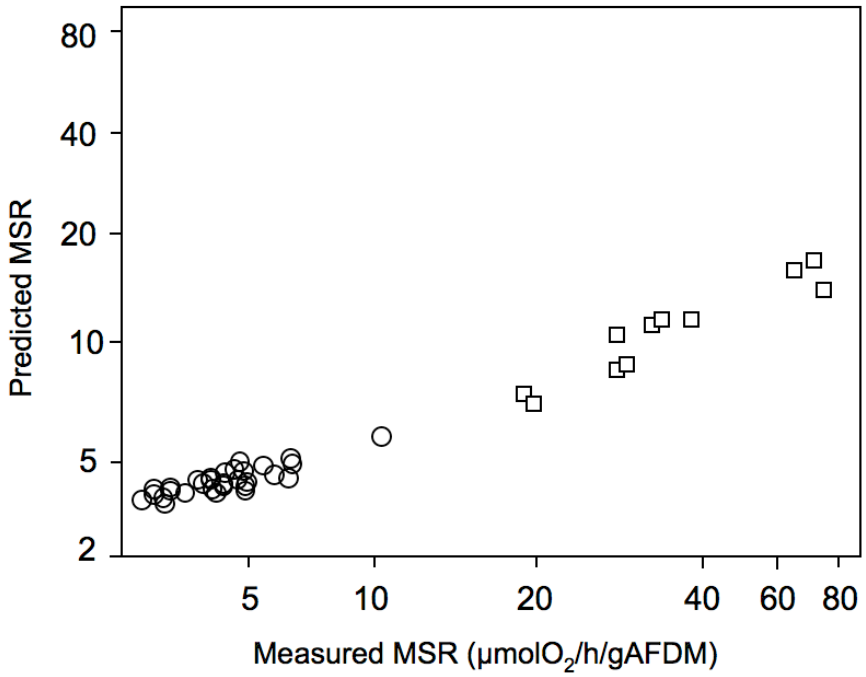

Fig 5: Comparison of reduced model estimates with measurements of *Arctica islandica* respiration published by Taylor and Brand (1975). Accuracy is <1 µmol below 20 µmol O_2 h^{-1} g^{-1}. Circles: measured by Taylor and Brand (1975); squares: measured by Bayne (1971) (*fide* Taylor & Brand 1975).

These findings contrast studies of shorter-lived bivalves, including data obtained for the blue mussel *Mytilus edulis*, which reaches maximum ages of up to 30 years. Sukhotin and co-authors documented decreasing respiration and filtration rates in *M. edulis* from a subarctic White Sea population above 6 years of animal age, indicating that blue mussels either lose or reduce respiratory capacities and water pumping activity over lifetime (Sukhotin and Pörtner 2001, Sukhotin et al. 2003). The present evidence suggests that in *A. islandica* MSR is independent of age for at least the first 100 years of lifetime. Most of the animals we have studied were younger than 100 years and the senescent decline of MSR may yet not have been detectable in our samples. However, as these cohorts seem to represent the most abundant age ranges in all populations they suffice for modeling of the overall metabolic capacities and energy demand in a given population.

Do different climatic adaptations in A. islandica populations affect the respiratory response to elevated temperature?

Temperature is assumed to be the most important environmental modulator of poikilotherm metabolism (Thompson 1984). Accordingly, the respiration model in Figure 3 shows mass specific metabolic activity of *A. islandica* to be significantly higher at higher temperature. Thermal adaptations in bivalves are best described by an Arrhenius model of temperature
dependence of metabolic rates (Peck and Conway 2000, Heilmayer and Brey 2003), and Q10 is a good summary descriptor of the temperature effect. There is evidence that bivalves become more temperature sensitive with increasing body mass (Bayne et al. 1976), i.e. Q10 may be linked to

body mass. Hence, differences in Q10 between SITEs may be partially caused by differences in size range between our population samples (see above). To evaluate this question, we reduced the whole data set to a common mass range from 0.3–6 g AFDM (n = 123) for all populations and built a new multiple linear model to predict MSR. The corresponding Q10 values, 4.88 for German Bight, 2.72 for Norway, 2.70 for White Sea, 1.22 for Kattegat and 1.37 for Kiel Bay did not provide a consistent picture: clipping of either very small animals (from Kiel Bay and White Sea population) or of very large animals (from German Bight, Norway population) both increased Q10. Thus, animal body mass differences can obviously not explain the observed differences in site-specific Q10 values. Instead, the strong interactions between SITE and temperature as well as body mass (Table 3) point towards site-specific adaptations in the physiological response to temperature. The exceptionally high value for the German Bight animals should be viewed with caution. A Q10 of 4.88 is quite high, particularly compared with the adjacent Kattegat.

Does AP affect respiration and do we see site or temperature specific differences in AP occurrence or duration?

Respiratory pauses (APs) did not affect long term (over hours) respiration in *A. islandica* (Fig. 2). Moreover, neither the percentage of AP-performing animals, nor the length of the APs differed between populations. We did not analyze the AP pattern of each specimen in detail, but the general picture is that of a non-rhythmic, very variable and individual behavior. Apparently, AP is not caused by external triggers, but reflects an internal behavioral pattern in the bivalves. Apneal behavior or to the contrary short bouts of elevated respiration have already been observed in other cold adapted bivalves, such as the Antarctic mud clams *Laternula elliptica* (Morley et al. 2007) and the protobranch *Yoldia eightsi* (Abele et al. 2001). There is a general trend in bivalves to keep mantle water PO_2 on low and protective levels (Abele et al. in prep.), and water breathers like *A. islandica* which live in the sediment water interface, must cope with fluctuant and up to normoxic oxygen levels in the inhaled water. Short bouts of accelerated or reduced respiration may contribute to the regulation of shell water PO_2. This behavior should not be confused with real metabolic rate depression (MRD), which represents a distinct and deliberate shut down of metabolic activity (Taylor 1976, Thompson 1984, Abele 2002, Strahl et al. 2007).

According to the rate of living-theory of aging (Pearl 1928), the MSR of the long-lived *A. islandica* should range at the lower end of the MSR range of bivalves. We compared 3583 respiration measurements from 58 different bivalve species compiled by Brey (2001), with 234 measurements of *A. islandica*, including the data of Taylor and Brand (1975) and our own measurements (Fig. 6).

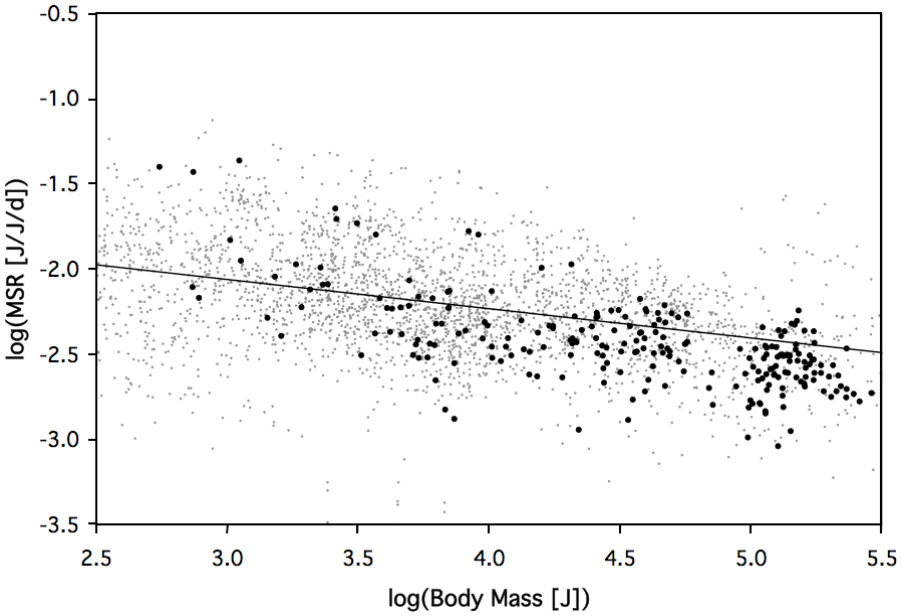

Fig 6: Temperature adjusted (10°C) mass specific respiration rate (MSR, in Joule Joule^{-1} day^{-1}) versus body mass (in Joules) in *Arctica islandica* (black dots, n = 234; Taylor and Brand, 1975 and own measurements) compared to data from 58 bivalve species (n = 3583). Straight line indicates regression of log(MSR) on log(M) over all data. MSR of *A. islandica* is significantly below bivalve average MSR ($P < 0.001$). For data, species and references see Brey (2001). To unify units of mass and respiration, original body mass data were converted to Joule (J) and respiration rates to Joule day^{-1} (J/d) (1 µmol O_2 = 46.8 J, Gnaiger 1983, and 1 mg AFDM = 21.82 J in bivalves, Brey 2001).

Regression of log (MSR, adjusted to 10°C) over log(M) indicates MSR of *A. islandica* to be significantly ($P < 0.001$) lower than the compiled bivalve MSR (Fig. 6). Note that the few isolated black dots showing higher respiration for *A. islandica* are from Bayne (1971), and do not fit the general *A. islandica* model. The overall low respiration in *A. islandica* indicates that the quahog belongs to the low tissue oxygenation-type, presumably keeping tissue PO_2 on extremely low levels during most of its burrowed and non-burrowing lifetime.

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PUBLICATION V

Age dependent patterns of antioxidants in *Arctica islandica* from six regionally separate populations with different life spans

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Abstract

Environmental factors such as temperature and salinity regimes shape lifespan in marine ectotherms. The question is whether or not the effect is through modification of metabolic ROS (reactive oxygen species) producing processes and thus in line with the Rate of Living – Free Radical Theory of Aging. We compared six biogeographically and climatically distinct populations of the extremely long-lived ocean quahog, *Arctica islandica*, for age-dependent differences in metabolic rates and antioxidant capacities (superoxide dismutase, catalase activity and total glutathione concentration). The temperature and salinity range covered by the sampling locations was 3.7–9.3 °C and 20–35 psu from the Norwegian coast, White Sea, Iceland, Kattegat, Kiel Bay and German Bight. Bivalve shells were used as age recorders by counting annual growth bands. Maximum determined age in different populations varied between 29 and 192 y. Extreme longevity observed in some North Atlantic *Arctica islandica* populations seems to be based on its very low lifetime mass specific respiration, in combination with stable maintenance of antioxidant protection over life in mature specimens. While the antioxidant capacity was similar between all populations, the shorter-lived populations exhibited highest metabolic rates and showed no metabolic response (Q_{10}) when warmed to higher temperature. Low and fluctuating salinity in the Baltic Sea may further exert a stress, which enhances respiration rates and shortens longevity in the Baltic Sea population. The exceptionally long lifespan of *A. islandica* cannot be exclusively explained by a well established antioxidant defense system, and the long lifespan of some populations may rather be a function of low ROS formation (low metabolic rate) and high damage repair/removal capacities.

Key words

Arctica islandica, longevity, antioxidants, metabolic rate, aging, temperature, salinity, inter-population variability

Introduction

Some marine bivalves are extremely long-lived, and among non-colonial organisms, the ocean quahog, *Arctica islandica*, has the longest confirmed lifespan (Ridgway and Richardson 2010). One individual with a lifespan of more than 400 years was reported (Wanamaker et al. 2008). According to the 'Rate of Living – Free Radical Theory of Aging' (Harman 1956, Pearl 1928), mitochondrial reactive oxygen species (ROS) formation links cellular oxygen consumption to individual lifespan. The argument is that higher metabolic oxygen consumption causes higher basal ROS production and faster accumulation of peroxidized metabolic intermediates, whereby accelerating the loss of cellular function and viability, and propelling cellular aging. ROS carry odd numbers of electrons in binding orbitals and are able to extract other electrons from lipids, causing lipid peroxidation, as well as oxidation of proteins and DNA molecules. Although the basic idea of ROS involvement in aging has been acknowledged in numerous studies for different model organisms and cell cultures, the issue is not as straight forward as was previously assumed. Recent studies on genetically manipulated mice failed to provide evidence for a connection between ROS generation, antioxidant capacity and lifespan (Pérez et al. 2009, Jang et al. 2009). Further, some species or higher taxa do not follow the rules of the 'Rate of Living – Free Radical Theory of Aging'. Especially many marine invertebrates have lower life expectancy than would be predicted from their low metabolic rates (Buttemer et al. 2010).

Studies comparing species of similar lifestyle from different environments to explain the modulating effect of environmental factors on aging are susceptible to confounding effects of genetic peculiarities, especially in invertebrate groups with long evolutionary history and large genetic distances between similarly looking animals. Hence, for these investigations, single species of broad geographical distribution seem to be promising models. Bivalve mollusks are applicable in biogeographical aging research (Abele et al. 2009, Philipp and Abele 2010). This group accommodates a high diversity of species-specific life-spans. Age determination of individuals can easily be done by counting annually forming growth bands in the bivalve shells (except for tropical species, which usually do not feature annual growth rings). In previous studies of cellular and organism aging, we could see that the 'Free Radical-Rate of Living Theory' principally applies, but different bivalve 'aging phenotypes' can be distinguished (Abele et al. 2009). Extremely long-lived and slow growing species, such as the ocean quahog *A. islandica*, display high antioxidant capacities (mainly antioxidant enzyme activities and glutathione redox buffer concentrations) and efficient cellular waste removal is maintained constant over an age range of at least 7-150 years (Strahl and Abele 2010). Contrary, some short-lived bivalves have developed physiological and biochemical strategies that retard the aging process (Philipp et al. 2006) until reproductive capacities are exhausted. These species often feature high growth rates and, repeatedly, mobilize and reallocate energy reserves between somatic growth and reproduction. Further, ROS producing and scavenging capacities are modified by climatic adaptation of metabolic rates in ectotherms and are specifically adjusted in ectotherms from different latitudes (Abele and Puntarulo 2004).

The aim of the present study was to compare biogeographically and climatically distinct populations of *A. islandica*, which differ with respect to population-specific lifespan. The ocean quahog colonizes areas with diverse environmental conditions in the Northern Atlantic Ocean, both along European and American coasts and genetic differences between populations are small (Dahlgren et al. 2000). Six geographically separated populations were included in our study. Determined individual maximum life span varies between 29 and 192 years across these populations (Begum et al. 2009), with especially old individuals encountered in an Icelandic population (Schöne et al. 2005a, Wanamaker et al. 2008). If antioxidants were indeed major factors supporting longevity, we should see far higher levels in populations with documented longer life spans. Further, if declining antioxidant capacity over lifetime is a major cause of aging, 30 years-old animals from the short-lived Baltic Sea population should have lower capacities than 100 years-old, and thus in respect to the MLSP physiologically younger, specimens from the long-lived Icelandic or North Sea populations.

Materials and methods

Animal and tissue sampling

A. islandica were collected from six different geographic locations covering a temperature and salinity gradient of 3.7 – 9.3 °C and 20 – 35 psu, respectively: Norwegian Coast (NC), Kattegat (KA), White Sea (WS), Kiel Bay (KB), German Bight (GB), and Iceland (IC) (Table 1). After collection, all animals were transported to the Alfred Wegener-Institute (AWI, Germany). They were kept several weeks at a temperature close to mean annual habitat temperature (Table 1) and salinity of the respective sampling site in 60 l flow-through aquaria with natural seawater and sediment, to recover from sampling and transportation stress. Bivalves were fed DT-live marine plankton (DT, USA, 1 ml ind^{-1} $week^{-1}$) once a week. Some animals from each population remained in the aquaria for four weeks prior to respiration measurements. The other animals were dissected directly within days after sampling, and pieces of mantle and foot tissues were snap frozen in liquid nitrogen for biochemical analysis.

Whole animal metabolic rate

Whole animal metabolic rates were measured as oxygen consumption at habitat temperatures (HT) and 5°C elevated temperature (ET) (Table 1) in a multi-channel modified intermittent flow system as described in Begum et al. (2009). The first subsample was maintained at HT, whereas the second subsample was acclimated stepwise (1°C temperature increase every 2 days) to ET. ET bivalves were kept for at least four weeks at the elevated temperature prior to respiration measurements. Animals that did not respire, or long periods of zero respiration were discarded. Respiration rates (R, μmol O_2 h^{-1} ind^{-1}) were determined from the slope of the oxygen saturation curve after subtraction of the microbial oxygen demand (chamber without animal). Percent O_2 saturation was transformed to O_2 volume concentration using known values of oxygen solubility (Benson and Krause 1984).

Table 1: Characteristics of sampling sites, temperature conditions and age range of mollusks used for respiration and antioxidants determination. (Depth: sampling depth, HT: acclimation habitat temperature, ET: 5°C elevated acclimation temperature). Mean annual temperature, salinity, and its amplitudes for each population around the depths where *A. islandica* lives were calculated from ICES internet database http://www.ices.dk.

Population	Location	Depth, m	Mean annual salinity (S), ppt	Amplitude of seasonal S variation ppt	Mean annual temperature (T), °C	Amplitude of seasonal T variation °C	HT, °C	ET, °C	Age range biochemistry (respiration), years
Norwegian Coast (NC)	69°39´N 18°57´E	10-30	34	0.7	6.4	5.6	4	9	54-80 (4-93)
White Sea (WS)	66°18´N 33°38´E	10-15	26	2.7	3.7	12.6	4	9	5-34 (3-53)
Iceland (IC)	66°02´N 14°51´W	14-22	35	0.4	4.1	6.2	5	10	5-192 (8-141)
Kattegat (KA)	56°10´N 11°48´E	33	32	2.5	8.0	6.7	8	13	15-39 (8-71)
Kiel Bay (KB)	54°32´N 10°42´ E	25	20-25	4.8	7.3	9.3	10	15	3-28 (4-29)
German Bight (GB)	54°09´N 07°47´E	40	33	1.1	9.3	11.6	10	15	34-125 (33-98)

$$R = \frac{\text{sat } t_o}{\text{sat } t_1} \cdot \alpha_{O_2} \cdot V_{Chamber} \qquad (1)$$

R: respiration rate (µmol O_2 h^{-1} ind^{-1}), α_{O_2}: oxygen solubility in sea water (µmol dm^{-3}), $V_{Chamber}$: volume of respiration chamber and tubing (dm^3), $\text{sat } t_o$: oxygen saturation (%) at the beginning of the experiment and $\text{sat } t_1$: oxygen saturation (%) after reduction of oxygen saturation till 70 % level of respiration in the closed system. Individual mass-specific respiration rates (MSR) were calculated according to:

$$MSR = \frac{R}{AFDM} \qquad (2)$$

MSR: mass specific respiration rates (µmol O_2 h^{-1} g^{-1}), R: respiration rate (µmol O_2 h^{-1} ind^{-1}), AFDM: ash free dry mass (g).

MSR was standardized to the average mean size of mollusks in all populations ($AFDM_{st}$ = 3.54 g) according to equation (3) using three-quarters power law, in which the metabolic rate is proportional to 0.75 power of body mass, which means the relation ln MSR to ln AFDM has slope b= –0.25 (McMahon, 1973).

$$MSR_{st} = e^{\ln MSR_{obs} + b(\ln AFDM_{st} - \ln AFDM_{obs})} \qquad (3)$$

MSR_{st}: mass specific respiration rates for standard size mollusk, MSR_{obs} observed mass specific respiration rate, $AFDM_{obs}$: observed individual ash free dry mass.

The effect of temperature on MSR was estimated, using the Arrhenius-Vant-Hoff's temperature

coefficient Q_{10}. We chose animals of similar size and age from each population at two temperatures and calculated MSR_{st} at two different temperatures – habitat and 5°C elevated:

$$Q_{10} = \left(\frac{MSR_{st\,ET}}{MSR_{st\,HT}} \right)^{\frac{10}{ET-HT}} \quad (4)$$

ET: elevated temperature (°C), HT: habitat temperature (°C), $MSR_{st\ ET}$: standard mass specific respiration at elevated temperature, $MSR_{st\ HT}$: standard mass specific respiration at habitat temperature.

In order to compare MSR in different populations (Table 1), MSR values were recalculated for the three temperatures 4, 9 and 14°C using the obtained coefficients Q_{10}:

$$MSR_{st\,t_2} = e^{\ln MSR_{st\,t_1} + Q_{10} \cdot (t_2 - t_1)/10} \quad (5)$$

$MSR_{st\,t_2}$: mass specific respiration rate at standard temperature (4, 9 or 14°C), $MSR_{st\,t_1}$: mass specific respiration rate at *in situ* temperature,Q_{10}: Q_{10} calculated from (4), t_1: *in situ* temperature, t_2: standard temperature.

Measurements of antioxidant activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured spectrophotometrically at 550 nm after Livingstone et al. (1992). 50-100 mg of foot tissue and 100-200 mg of mantle tissue were ground in liquid nitrogen and homogenized in Tris buffer (20 mM TRIS-HCl, 1 mM EDTA, pH 7.6) 1:30 (w:v), using a glass homogenizer. Samples were centrifuged for 3 min at 12000 g at 4 °C. SOD activity is measured as degree of inhibition of the reduction of cytochrome *c* by $O_2^{\bullet -}$ generated by a xanthine oxidase/xanthine system in 43 mM potassium buffer with 0.1 mM EDTA, pH 7.8, T = 20 °C. One unit of SOD inhibits the reduction of cytochrome *c* by 50 % under the assay conditions. Mitochondrial and cytosolic SOD isoforms were not distinguished.

Catalase (Cat EC 1.11.1.6) activity was determined after Aebi et al. (1984). 50-100 mg of foot tissue, 100-200 mg of mantle tissue were each ground in liquid nitrogen and homogenized 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 1:6 (w/v). Samples were centrifuged at 12000 g for 15 min at 4 °C. The activity was determined by recording the time of H_2O_2 decomposition, resulting in a decrease of absorption from 0.45 to 0.40 at 240 nm, T = 20 °C.

Total glutathione (GSH+2GSSG) was measured after Griffith (1980) and Tietze (1969). Samples of 50-80 mg for foot and 50-100 mg for frozen mantle tissue were ground in liquid nitrogen and homogenized using a pre-cooled glass homogenizer in ice-cold 5% sulfosalycilic acid (previously bubbled with nitrogen gas) 1:10 (w/v). The homogenate was bubbled with N_2 gas for 30 seconds and then centrifuged for 5 min at 15000 g at 4°C. The sulfhydryl group of GSH reacts with DTNB (5,5 '-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). Oxidized glutathione disulfide (GSSG) is reduced by NADPH in the

presence of glutathione reductase. Assay conditions were: 0.3 mM NADPH, 6 mM DTNB and 50 U/ml GR in 125 mM potassium phosphate buffer containing 6 mM EDTA (pH 7.2). The rate of formation of TNB was measured at 412 nm, and GSH quantified by reference to a standard curve.

Citrate synthase (CS, EC 4.1.3.7, key enzyme of the citric acid cycle) activity was measured after Sidell et al. (1987). 80-100 mg of foot and mantle tissue were each ground in liquid nitrogen and homogenized with a glass homogenizer (Nalgene) in Tris-HCl buffer (20 mM Tris-HCl, 1 mM EDTA, 0.1 % Tween® 20, pH 7.4) 1:8 (w/v). Homogenates were sonicated on ice for 15 min in a Branson Sonifier 450 (output control 8, Duty cycle 50 %), and centrifuged for 5 min at 7400 g and 0°C. CS activity was measured recording the absorbance increase of 5 mM DTNB (5,5'dithiobis [2-nitrobenzoic acid]) in 75 mM Tris HCl (pH 8.0), 0.4 mM acetyl-CoA and 0.4 mM oxaloacetat at 412 nm. Activity was calculated using the extinction coefficient ε_{412} of 13.61 mM^{-1} cm^{-1}. Enzymes activities were expressed in U g^{-1} wet mass (WM), and glutathione concentration was calculated as nmol g^{-1} WM.

Total antioxidant capacity was calculated for each individual in all populations as the percentage of each antioxidant (CAT, SOD, tGSH) of the maximal capacity measured for this particular antioxidant in the whole survey.

Individual age determination

Individual age of each animal used in the respiration experiments and all biochemical measurements was determined by counting the shell growth bands as described in Begum et al. (2009).

Statistics

Mass specific respiration rate, enzyme activity (U g^{-1} wet mass (WM); CS, SOD, CAT) and total glutathione concentration (tGSH, nmol g^{-1} WM) may be affected by age, tissue type and population. The effect of age on the measured traits was tested by regression analyses. We tested for differences between populations by ANOVA (dependent variable vs population by tissue type) and post-hoc Tukey HSD test after the Levene test for homogeneity of variances. If the Levene test was significant, we used nonparametric Kruskal-Wallis ANOVA. Differences between tissues within one population were tested by Mann-Whitney U test.

Results

Mollusks belonging to six populations were assigned to two climatic groups – 'cold adapted' (Norwegian coast, Iceland, White Sea, acclimated to lower temperatures of 4-5°C, Table 1) and 'warm adapted' (Kattegat, Kiel Bay, German Bight, acclimated to higher temperatures 8-10°C, Table 1).

Metabolic Rates

Since the age composition of mollusk samples differed significantly between populations (see Table 1), the effect of age on MSR was tested for the whole sample batch. As no significant age dependence of MSR was observed (regression analyses, $P > 0.05$) the populations could be compared without accounting for an age effect. Among the cold adapted *A. islandica*, bivalves from the Norwegian coast had the highest MSR at both acclimation temperatures when compared to Iceland and White Sea bivalves (Fig. 1, white and grey bars), which had similar and lower MSR. Mollusks from the warm climatic group did not differ in MSR at the high temperature of 14°C (Fig. 1, black bars). At the intermediate acclimation temperature of 9°C, the average MSR of mollusks from the German Bight (GB: 2.4 µmol O_2 h^{-1} g^{-1} AFDM) was significantly lower than in the Kattegat (KA: 6.3 µmol O_2 h^{-1} g^{-1} AFDM, Fig. 1). KA and KB mean MSRs did not differ. MSRs of the mollusks from KB and KA populations were almost indifferent to warming, and the respiratory Q_{10} was 1.1 at both sites (Fig. 2). Of all populations, Kiel Bight and Kattegat animals are most adapted to variable environmental conditions not only, but primarily, with respect to temperature and salinity. The other four populations were significantly more responsive to high temperature acclimation. Mollusks from the IC and GB populations were highly temperature sensitive, their metabolic rates doubling in response to a thermal increment of 5°C (Q_{10} values were 5.8 and 5.0, respectively, Fig. 2). *A. islandica* from the WS and NC increased metabolic rate 1.5 times at elevated temperature (Q_{10} = 2.4, Fig. 2) and appeared a little less sensitive to thermal challenge.

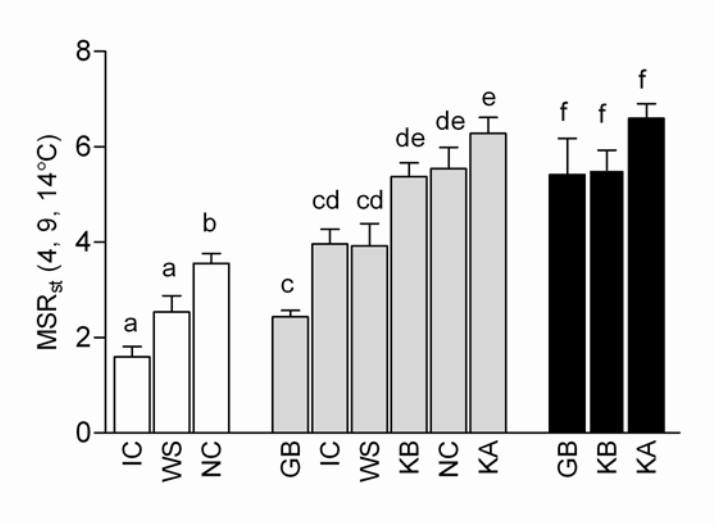

Fig. 1. Mass specific respiration rate standardized to mean size mollusks (3.54 g AFDM) at 4 °C (white bars), 9 °C (grey bars) and 14°C (black bars) in Iceland (IC), White Sea (WS), Norwegian coast (NC), German Bight (GB), Kiel Bay (KB) and Kattegat (KA) populations of *A. islandica*. Data are mean ± S.E. expressed as MSR_{st} (4, 9, 14°C), µmol O_2 h^{-1} g^{-1} AFDM as average over lifetime. Significant differences between populations at each standard temperature (ANOVA, $P < 0.05$) shown with different letters (a, b, c, d, e, f).

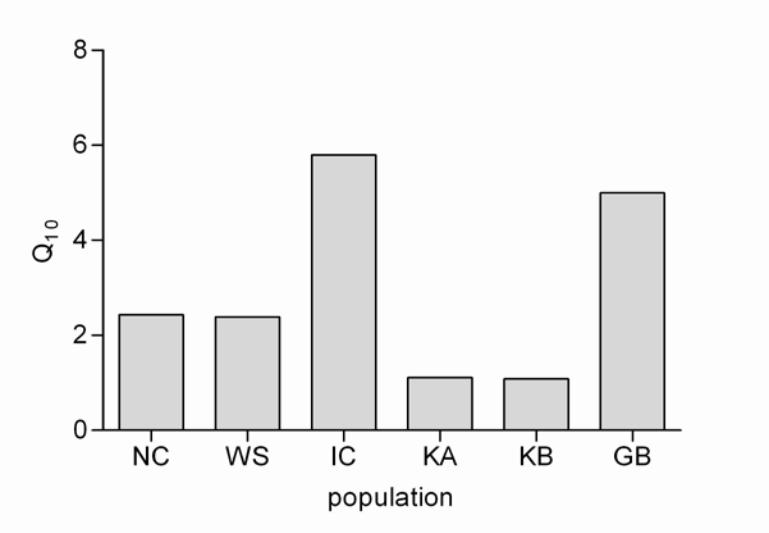

Fig. 2. Q_{10} in Norwegian coast (NC), White Sea (WS), Iceland (IC), Kattegat (KA), Kiel Bay (KB) and German Bight (GB) populations of *A. islandica*.

Citrate synthase and antioxidant enzyme activities over age

In the majority of populations, enzyme activities did not change with age of the mollusks (individual antioxidant x population, Table 2). CS activity was studied only in animals from the German Bight where values remained stable over lifetime in foot and mantle, the only tissues we tested. The age influence on antioxidants differed between tissues. In mantle tissue, a negative correlation with age was observed for SOD activity in Iceland *A. islandica* and for CAT activity in German Bight and young immature Iceland animals. tGSH concentration decreased with age in immature Iceland animals (Table 2; data from the Iceland population were taken from Abele et al., 2008, and included into the regression analyses). To the contrary, in foot muscle an age-related increase of SOD activity in bivalves from Kiel Bay and the White Sea, and of CAT activity in Kattegat bivalves, was observed (Table 2).

Table 2. Relation between SOD, CAT, tGSH and CS activities and age in different populations of *A. islandica*. Significance level: $P < 0.05$. + positive correlation with age, – negative correlation with age, no – correlation beyond significance level, grey fields: no data, y = years.

	SOD	CAT	tGSH	CS		SOD	CAT	tGSH	CS
Population	*Mantle*					*Foot muscle*			
NC	no	no	no			no	no	no	
WS	no	no	no			+	no	no	
IC	–	– (< 33 y) no (> 33 y)	– (< 33 y) no (> 33 y)						
KA	no	no	no			no	+	no	
KB	no	no	no			+	no	no	
GB	no	–	no	no		no	no	no	no

Antioxidant capacities in different tissues

The different components of the antioxidant system were distinctly weighted between *A. islandica* mantle and foot tissue (Table 3). Across populations, SOD activity and total glutathione (tGSH) concentration were significantly higher in foot than in mantle tissue (Mann-Whitney U test, $P < 0.05$, Fig. 3 (a), (c), Table 3) (except total glutathione concentration in the White Sea mollusks). Contrary, CAT activity was always significantly higher in mantle than in foot tissue (Mann-Whitney U test, $P < 0.001$, Fig. 3 (b), Table 3). To check whether the higher SOD activity in foot tissue may relate to higher mitochondrial density, we exemplarily measured the activity of the mitochondrial marker citrate synthase (CS) in both tissues of the German Bight individuals. Indeed CS activity was slightly higher in foot than in mantle tissue, but the differences did not reach significance (Fig. 3 (d), Mann-Whitney U test, $P = 0.27$, Table 3).

Table 3. Activities of superoxide-dismutase (SOD), catalase (CAT), total glutathione concentration (tGSH), citrate synthase (CS) and total antioxidant activity in foot and mantle tissues in different populations of *A. islandica*. Data are mean ± S.E. expressed as U g^{-1} WM as average over lifetime except tGSH expressed as nmol g^{-1} WM and total antioxidant activity expressed as % of maximum antioxidant activity, n = number of individuals, – no data.

Population	*Tissue*	SOD	n	CAT	n	tGSH	n	CS	n	Total anti-oxidants	n
NC	*Foot muscle*	2293.8 ± 194.5	10	598.8 ± 40.0	10	673.5 ± 86.7	10	–	–	59.3 ± 3.3	30
WS		1795.4 ± 132.4	19	505.8 ± 29.5	18	548.1 ± 60.2	7	–	–	50.0 ± 2.2	44
KA		2535.0 ± 143.0	10	814.7 ± 26.8	10	768.3 ± 73.4	10	–	–	71.3 ± 3.1	30
KB		1765.8 ± 116.1	17	683.5 ± 38.0	13	1055.9 ± 88.8	8	–	–	63.3 ± 3.1	38
GB		2184.8 ± 112.7	10	783.5 ± 36.6	10	701.3 ± 44.1	10	2.60 ± 0.07	8	65.2 ± 2.9	30
Mean		2034.4 ± 71.2	66	655.1 ± 21.5	61	749.2 ± 39.2	45	–	–	60.9 ± 1.4	172
NC	*Mantle*	1235.8 ± 80.6	8	3097.3 ± 258.8	10	401.4 ± 41.3	10	–	–	54.5 ± 4.0	28
WS		578.3 ± 23.7	24	2150.3 ± 124.7	28	404.9 ± 63.2	8	–	–	36.0 ± 1.4	60
IC		662.2 ± 16.6	37	3212.1 ± 158.9	41	419.3 ± 40.2	33	–	–	45.3 ± 1.6	111
KA		1137.7 ± 59.1	9	3094.1 ± 250.0	10	406.8 ± 77.4	10	–	–	53.6 ± 4.0	29
KB		863.0 ± 69.0	19	2543.3 ± 168.9	20	741.8 ± 30.0	15	–	–	55.1 ± 2.8	54
GB		866.7 ± 74.7	10	2618.3 ± 261.5	10	462.1 ± 39.0	10	2.22 ± 0.27	10	47.8 ± 2.7	30
Mean		781.0 ± 26.4	107	2780.4 ± 86.0	119	475.6 ± 24.2	86	–	–	47.1 ± 1.0	312

Antioxidant capacities in different populations

SOD activity in both, foot and mantle tissues were generally higher in Kattegat, German Bight and Norwegian coast bivalves (in fully saline habitats), than in Kiel Bay and White Sea where animals live in brackish water, although not all differences were significant (Fig. 3 (a)). The only exception was the fully marine, long-lived Iceland population (Abele et al. 2008) in which mean SOD activity was as low as in White Sea animals (only mantle tissue was measured and could be compared in IC animals). Further, WS animals had the lowest CAT activity of all populations in both tissues. Similarly low CAT activity was recorded in Norwegian animal foot muscle, whereas mantle tissue of NC specimens did not differ from the other populations and was higher than in WS animals (Fig. 3 (b)). *A. islandica* from Kiel Bay had significantly higher tGSH concentration in mantle and foot compared to all cold-adapted populations (IC, NC and WS). The cold-adapted populations had

similar and lower tGSH concentrations in both tissues. Of all populations, the WS animals featured the lowest overall antioxidant capacity in mantle tissue and, together with NC animals, displayed the lowest antioxidant capacities in foot muscle (Fig. 3 (e)).

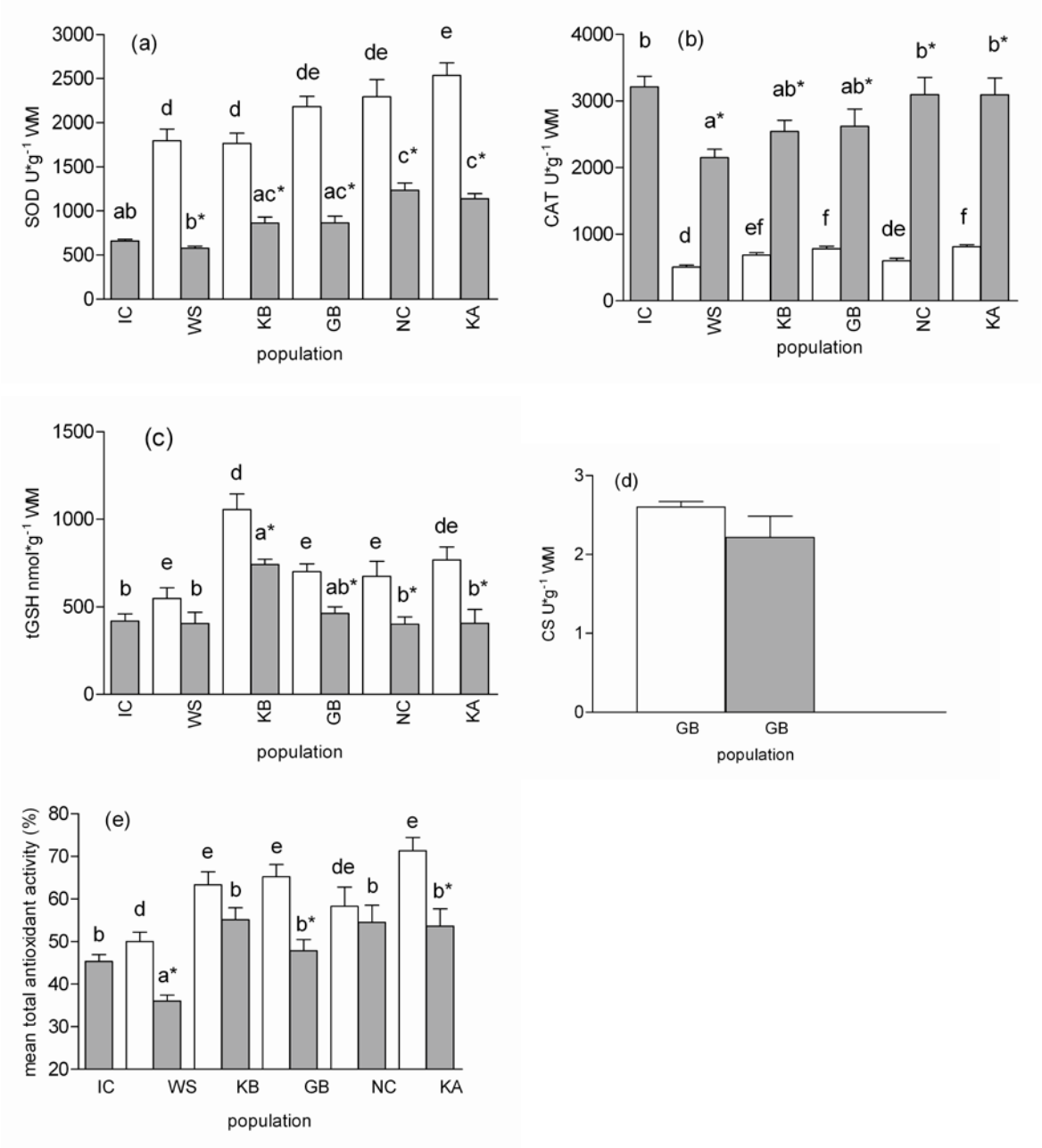

Fig. 3. Activities of superoxide-dismutase (SOD) (a), catalase (CAT) (b), total glutathione concentration (tGSH) (c), citrate synthase (CS) (d) and total antioxidant activity (e) in foot (white bars) and mantle (grey bars) tissues in Iceland (IC), White Sea (WS), Kiel Bay (KB), German Bight (GB), Norwegian coast (NC), and Kattegat (KA) populations of *A. islandica*. Data are mean ± S.E. expressed in SOD, CAT and CS as U g^{-1} WM as average over lifetime, in tGSH as nmol g^{-1} WM and in total antioxidant activity as % of maximum antioxidant activity. Populations annotated with the same letter (a, b, c – mantle tissue; d, e, f – foot tissue) do not differ significantly. Stars show significant ($P < 0.05$) differences between foot and mantle tissues within the population.

Discussion

Can life-long high antioxidant protection explain extreme longevity of A. islandica?

The exceptional long lifespan in the bivalve *A. islandica* cannot be sufficiently explained by a better-established antioxidant defense system. Indeed, individual enzyme activities and tGSH range among, but not above the highest values observed in mantle and foot tissues in other bivalves with shorter lifespan such as *Crassostrea virginica*, *Mya arenaria*, *Laternula elliptica, Adamussium colbecki*, *Mytilus edulis, Pecten jacobaeus* and *P. maximus* (Abele et al. 2008, Ballantyne and Berges 1991, Gamble et al. 1995, Philipp et al. 2005 a, b, 2006, Sukhotin et al. 2002, Viarengo et al. 1991, 1995). However, in the presence of the same amount of antioxidants, ROS production by *A. islandica* mitochondria may be lower, either because of its extremely low metabolic rate (Begum et al. 2009), or because of more efficient mitochondrial electron transport in its mitochondria. Indeed, a recent summary of *in vitro* ROS formation in mitochondrial isolates from different bivalve species indicates *A. islandica* to produce less mitochondrial ROS per mg of mitochondrial protein than other mud clams (*Mya arenaria, Laternula elliptica*) in respiratory states 3 and 4 under *in vitro* conditions (Buttemer et al. 2010). Thus, a well-established antioxidant defense, together with low species-specific metabolic rates and low basal mitochondrial ROS formation altogether pose a strong argument for extended longevity of *A. islandica.*

An age effect on metabolic rate of *A. islandica* was not observed in our study. A general decrease of mass specific metabolic rate in the course of ageing is reported for homeotherms and insects (see for review McCarter 1995), however, with few exceptions (O'Connor et al. 2002). Especially for species with infinite growth, such as bivalves, it is difficult to distinguish between the effects of size and age, which are strongly correlated factors. Studies of mere age effects on metabolic rates in these species are quite rare, and the results are controversial. A decrease of respiration rate with age has been reported in fish *Cichlasoma nigrofasciatum* (Fidhiany and Winckler 1998) and in the bivalves *Crenomytilus grayanus* (Zolotarev and Ryabushko 1977), *Argopecten irradians irradians* (Bricelj et al. 1987) and *Mytilus edulis* (Sukhotin and Pörtner 2001, Sukhotin et al. 2006). However, in other studies this effect was poor or not seen at all (Pérez Camacho et al. 2000, Sukhotin et al. 2002). In the present study the age dependent effect might however be obscured by the combination of the low metabolic rate, high inter-individual variability and the relatively low amount of data at older ages where such an effect would most likely be visible and also more or less size-independent.

A decline of antioxidant defense (catalase, glutathione) in aged animals is commonly related to advanced aging and has been detected in bivalve mollusks such as oysters (Ivanina et al. 2008), clams and scallops (Philipp et al. 2005a, 2006, 2008). Shorter-lived species (*Aequipecten opercularis* and *Mya arenaria*) display a faster increase of oxidative damage marker concentrations, together with a decrease in antioxidants over age when compared to longer-lived bivalves of similar lifestyle (Antarctic species *Adamussium colbecki* and *Laternula elliptica*, Philipp et al. 2005 a, b, 2006). We found an age-related decline of SOD activity in mantle tissue of *A. islandica* from the

long-lived IC population, a decline in CAT activity (in young, not yet mature IC and GB mollusks), and a decline in total glutathione concentration in immature IC mollusks (<32 years of age). In sexually mature IC animals >30years, glutathione and CAT values did not change up to 150-190 years of age (Table 2, Abele et al. 2008). The allometric decline of specific metabolic rate with increasing size within the phase of active growth, observed in *A. islandica* as in other mollusks, is supposedly tantamount to a reduction of free radical generation by the mitochondria due to the decrease in metabolic rate with increasing size. This may reduce the need for energetically costly production of antioxidant enzymes in larger specimens, as observed in mantle tissue of *A. islandica* from some populations. SOD activity increased in foot tissue of KB and WS populations and also CAT activity in KA bivalves increased over age (Table 2). The upward trend of antioxidants with age is rarely observed in ectotherms. SOD and glutathione peroxidase increased with age in the shrimp *Aristeus antennatus* (Mourente and Diaz-Salvago 1999), and glutathione concentration was higher in older than in younger *Oncorhynchus mykiss* (Passi et al. 2004). This trend may indicate the increased demand in antioxidants at advanced age but could also be an effect of sampling the 'late survivors'. These especially longest-lived individuals within a population are supposed to be endowed with above average SOD or CAT activities from the beginning on, and thus better protected from oxidative stress than their average congeners. As the relative abundance of these well protected 'late survivors' increases within older cohorts, antioxidants seemingly increase over age in a population. However, KA and WS populations also feature the shortest maximum lifespan (MLSP) (Table 1) and experience the most intense environmental variability and the lowest habitat salinity. Thus, increasing antioxidants over population lifetime may indeed indicate physiological adjustments to elevated environmental stress in these populations. Nevertheless, within the age ranges studied in all six populations, we never detected a significant age influence on metabolic rate (MSR), or a consistent effect of age on antioxidant activities within one tissue across all populations (Table 2). We therefore conjecture that *A. islandica* are able to maintain MSR and mitochondrial ROS production low and stable over most of their lifetime and, at the same time, preserve constant levels of antioxidants into late age, but probably not induce the levels in aged specimens. *A. islandica* are indeed of low mobility, low metabolic rates and avoid too high tissue oxygenation by keeping PO_2 in their mantle cavity fluid far below saturation level (Abele et al. 2010). Frequent events of self-induced metabolic rate depression of *A. islandica* (Taylor 1976), which were not included in our MSR-results, are bound to significantly reduce even these very low measurable oxygen consumption and ROS production rates over lifetime under *in situ* conditions. Together, all these factors support the exceptionally long lifespan of the ocean quahog.

Tissue specific antioxidant patterns.

The antioxidants are distinctly weighted in *A. islandica* mantle and foot tissues: 2-times higher SOD activity and tGSH concentrations in foot than in mantle contrast 4-times higher CAT activity in mantle than foot tissue. Mantle is a respiratory tissue in bivalves, and the higher CAT activity may be targeted to the higher O_2 concentrations in the mantle and detoxify ROS in actively respiring animals. In an earlier paper investigating only the Iceland population, we observed even higher SOD and CAT activities and tGSH concentration in *A. islandica* gills, than in mantle tissue (Abele et

al. 2008). This is in keeping with the extension of the gills into the surrounding seawater in ventilating animals, whereas the mantle, although a respiratory surface, is located within the bivalve shell and mostly bathed in lowly oxygenated mantle cavity water (Abele et al. 2010). By contrast, the foot is a massive organ with relatively low surface area, either contracted into the shell, or extended into the sediment for burrowing movements and, thus, better protected from contact with oxygenated water. Foot muscle mitochondria may produce more $O_2\bullet^-$ during burrowing exercise. This $O_2\bullet^-$ is converted to H_2O_2 by SOD and further detoxified by catalase and the glutathione system. High antioxidant activities and glutathione levels are common in exercising muscle and glutathione levels in human skeletal muscle increase during exercise training (Powers et al. 1999).

Between-population comparison

Maximum lifespan in natural populations is often underestimated because its determination depends largely on contingency with respect to the chance of sampling the 'oldest' individuals in a population (Philipp and Abele 2010). Therefore, reported maximum life spans in wild populations should always be viewed with caution (Beukema 1989). However, MLSP of *A. islandica* clearly differs between geographically separated populations. In the present study, the oldest specimens of 150-190 years were exclusively from the IC population, which is in agreement with reports of extremely old animals occurring in this region (Schöne et al. 2005 b, Wanamaker et al. 2008). Next, the animals from the GB population are relatively long-lived, and the oldest animal we encountered was 125 years old. WS (<55 years) and KB (<30 years) populations had the shortest life spans in our study, and animals of older age have never been reported from either area. *A. islandica* from IC and GB populations displayed the lowest MSR of all populations (Fig. 1) in spite of adaptation to different mean habitat T: cold in IC and warm-temperate in the GB, and different, although overlapping, annual temperature windows (Table 1). Narrower thermal windows and limited food supply during winter at higher latitudes have been suggested to reduce metabolic rates and favor burrowing and metabolic rate depression during winter (Buick and Ivany 2004, Strahl and Abele 2010), which might support extreme longevity at IC.

Hypothetically, thermal sensitivity of metabolism may also change over age in *A. islandica*. Thus, the observed inter-population differences might be explained by the age composition of the samples, i.e., IC and GB populations are represented by older animals than KB and KA populations. To disclose a potential age effect on thermal sensitivity (reflected by Q_{10}), animals of similar age were grouped in each population, resulting in 2 to 4 groups per population and Q_{10} values calculated for each age group (from HT and ET in Table 1). Plotting the resulting data against the mean age in each group revealed a significant age-associated increase of Q_{10} values (Fig. 4). This rather simple analysis suggests that indeed older animals seem to be more sensitive to warming. Higher respiratory Q_{10} in the long-lived IC and warm adapted GB populations, together with their low temperature specific respiration rates at 4°C (IC) and 9°C (GB) are indicative of their high thermal sensitivity. By comparison, shorter-lived *A. islandica* from KB and KA were characterized by very low temperature dependence reflected by Q_{10} values. This may be due to the fact that the animals were warmed already beyond maximum habitat temperatures and thus were not responding to the thermal

increase. However, there was no sign of stress in these groups such as accelerated death rates, so we assume that thermal stress was still minimal. Alternatively, higher basal MSRs in the population most strongly exposed to environmental fluctuations with respect to salinity and temperature (KB, KA and WS) confers more flexible response to thermal challenge (see also Clarke and Frazer 2004), changes in protein expression and better adjustable mitochondrial energetic coupling (Keller et al. 2004).This flexibility is acquired at the expense of population life-span. In other words: wider thermal and salinity windows in KA, WS and KB cut on population life expectancy, as shown in Fig. 5. This causes *A. islandica* to adopt a new life strategy in 'low and variable salinity environments', in which more intense and perhaps earlier reproduction as well as adjustment to constantly changing environmental conditions enhances MSR and shortens lifespan.

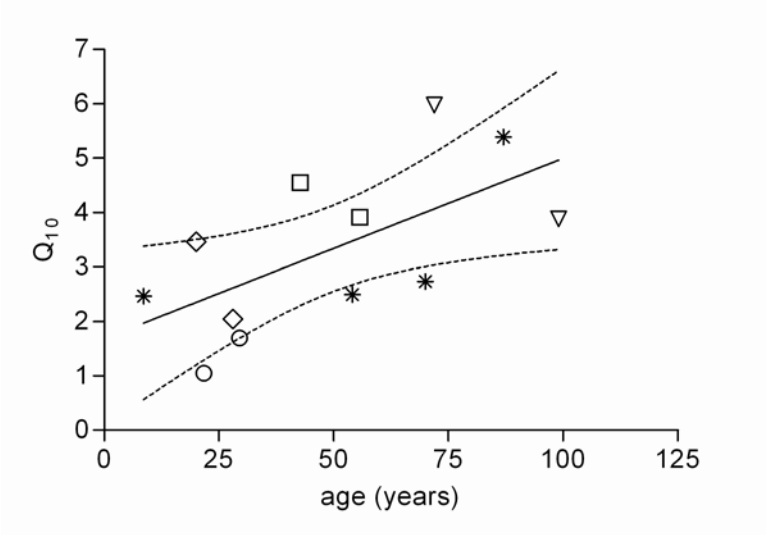

Fig. 4. Q_{10} versus age (age, y) in Norwegian coast (stars), White Sea (diamonds), Iceland (reversed triangles), Kattegat (circles) and German Bight (squares) populations of *A. islandica*. Straight line indicates regression of Q_{10} on age (Q_{10}=1.6869+0.0331*age, n=12, p=0.03). Animals of similar age were grouped in each population, resulting in 2 to 4 groups per population and Q_{10} values calculated for each age group (from HT and ET in Table 1)

This is certainly not a genetic trait, but a slowly evolved phenotypic adjustment in response to more challenging/costly environmetal conditions. The high thermal sensitivity retained in the GB *Arctica* population may already pose a problem. The population currently experiences a widening of the annual temperature range including summer and winter extremes, and a conspicuous warming trend (Wiltshire and Manley 2004), which is connected to global climate change. It further suffers additional impact from fishery and eutrophication (Rijinsdorp and van Leeuwen 1996, Rumohr and Kujawski 2000). As a consequence, longevity is shorter than in the IC region, and young recruits of less than 30 years are absent in the GB.

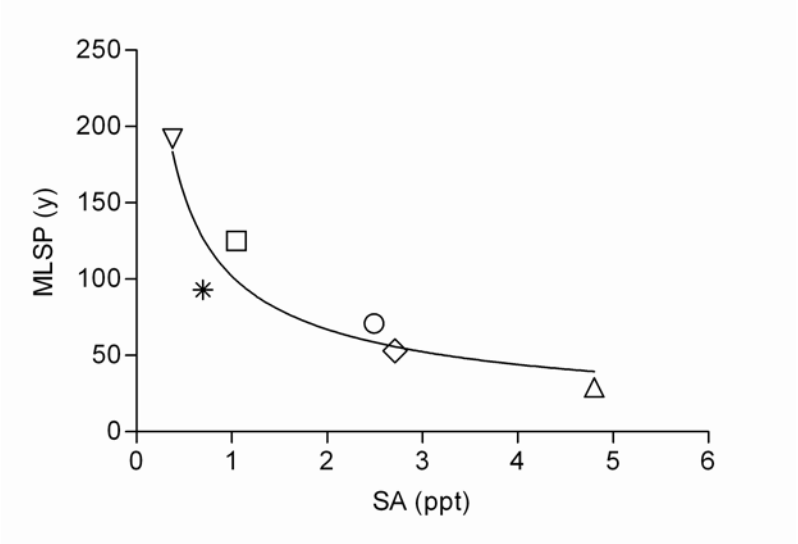

Fig. 5. Population specific maximal lifespan (MLSP, y) versus salinity amplitude (SA, ppt) in Norwegian coast (stars), White Sea (diamonds), Iceland (reversed triangles), Kattegat (circles) and German Bight (squares) populations of *A. islandica*. Best-fit line indicates regression of MLSP on salinity amplitude ($MLSP=102.1*SA^{-0.606}$, n=6, p=0.0063).

Antioxidant activities in different populations of *A. islandica* are much more homogenous than expected from their vastly differing MLSPs. Especially the total antioxidant capacities are similar in most cases and only slightly lower in WS specimens (Fig. 3e). This intra-specific study of *A. islandica* populations clearly documents that antioxidant levels do not correlate with lifespan, and hence these major ROS scavengers presumably support, but do not *cause* extreme longevity in the Icelandic population. Instead, total antioxidant capacity, CAT and SOD activities align with the mean annual habitat temperature recorded in each population habitat (see Tables 1 and 3). This underlines that metabolic ROS formation increases at higher temperatures, and that ectotherms have to enhance the levels of antioxidants and presumably other stress proteins to counteract thermal increase of ROS levels. Thus, enhanced antioxidants in warmer populations of the biogeographic distribution gradient balance oxidative stress, but do not increase life expectancy. This indicates that these organisms adjust their life history strategy to the environmental specifications, and express only so many antioxidants/stress proteins as required under these conditions. We conclude that population specific MLSP in long-lived, relatively inactive and sessile marine ectotherms is subject to environmental forcing.

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PUBLICATION VI

Aging in marine animals

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A general overview of free radicals and aging

The process of aging is defined as the progressive deterioration of cells, tissues and organs, associated with a decline in physiological function over time. Organisms show vastly different maximum life spans (Table 1) and the question why and how we age has always fascinated researchers. Up to now over 300 aging theories were formulated (Medvedev 1990). Some of the older theories have already been disproved 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.

Aging theories can be categorized in evolutionary and mechanistic theories. Evolutionary aging theories ask the question why aging occurs at all, while mechanistic aging theories ask which systemic and cellular changes are happening during the aging process and lead to a decline in performance and fitness i.e. what are the underlying mechanisms of the aging process. The currently most popular evolutionary aging theories are the 'Mutation accumulation' theory and the 'Antagonistic pleiotropy or trade-off theory'. Both theories predict that genes exist that have a negative influence on fitness and performance only in old but not in young animals, or, like a Janus head, even have a beneficial effect (pleiotropy) at young ages. Mechanistic aging theories investigate the reasons of the observed decline in performance and fitness with age. Current investigations indicate that stress-resistance mechanisms like resistance against cellular damage and increased repair capacities, but also cellular signaling pathways, such as the insulin pathway and, further, the level of nutrition in terms of calorie uptake per time in an individual (caloric restriction) are main players in the determination of individual lifespan (Gems and Partridge 2008). Some interactions between these players, however, are still not resolved.

Aging and free radicals: a general overview

The high potential of reactive oxygen species (ROS) to cause oxidative damage led Denham Harman (1956) to suspect ROS of being involved in the aging process. In 1956 he formulated one of the most well-known but also heavily criticized aging theories, the 'Free Radical Theory of Aging'. The theory has since been modified into the 'Oxidative Stress Theory of Aging' because some oxygen species like peroxides, which are formally not free radicals, also play a role in the oxidative damage of cells. The theory states that ROS are produced by all aerobic organisms as by-products of normal metabolism. Due to their destructive action, damage of cellular structures gradually accumulates over the lifespan of an organism, leading to mitochondrial and cellular degradation and disturbances of physiological processes during aging. Oxidative damage can directly interfere with molecular function and hence impair important cellular processes (gene transcription, metabolism, signal transduction), but can also lead to a nonspecific decline in cellular function caused by the accumulation of damaged molecules (macroaggregrates, lipofuscin) (Brunk and Terman 2002).

ROS are primarily produced in mitochondria; hence these organelles may play a major role in aging. Mitochondrial structures are especially prone to oxidation due to their close vicinity to the major generation site of free radicals, the respiratory chain, located in the inner mitochondrial membrane. Further the mitochondrial DNA (mtDNA) is located close to the inner mitochondrial membrane and lacks histones which can protect the DNA against oxidative damage and thus mitochondrial DNA experiences higher oxidative damage compared to nuclear DNA (Richter et al. 1988; Yakes and Van Houten 1997). Mitochondrial damage can finally drag the cell into a 'vicious cycle' with a positive feedback when mitochondria, already damaged by ROS, produce free radicals at greater intensity. The 'Mitochondrial theory of aging' is a refined version of the 'Oxidative stress theory of aging' and puts the mitochondria into focus of the aging process (Harman 1972). The generation of these ROS is balanced by the activity of antioxidant enzymes and small molecular antioxidants (e.g., glutathione, vitamins E, C and coenzyme Q), which remove ROS, stop chain reactions and protect cellular organelles, membranes and macromolecules from oxidation.

According to the theory, aging in animals is usually associated with a progressive misbalance between ROS production causing 'oxidative stress', an expression describing ROS-mediated deleterious processes such as oxidative damage of vital molecules, like lipids, proteins and DNA, and cellular defense and repair systems. This process is accompanied by the accumulation of various intermediate and end products of ROS-induced chain reactions. Some of these products are used for assessment of oxidative damage in cells: ketones and aldehydes, including malondialdehyde (MDA), and 'fluorescent age pigments (FAPs)', including lipofuscin and representing the most frequently measured markers of lipid peroxidation. Carbonylated proteins are used for quantification of protein degradation and 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 2,6-diamino-4- hydroxy- 5-formamidopyrimidine (FapyGua) represent biomarkers of nucleic acid damage by ROS (see chapter Grüne).

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, were investigated to a great extent 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). Several observations corroborate the 'oxidative stress theory of aging'. It is generally found that lipid and protein damage as well as DNA damage increases with increasing age (see technical section for aging markers below) (Shigenaga et al. 1994; Perez-Campo et al. 1998; Barja 1999; Barja 2004). Many long lived organisms show reduced oxidative damage or increased resistance to oxidative stress compared to shorter-lived comparable species. In mutant mice (*Mus musculus*), flies (*Drosophila melanogaster*) or worms (*Caenorhabditis elegans*) an increased lifespan is found to correlate with higher resistance to oxidative stress or lower oxidative damage. Prominent mutants are the Snell and Ames dwarf mice (Liang et al. 2003), the *age-1* and *daf-2 Caenorhabditis* mutants (Ruvkun et al. 2010), and *mth* (*methuselah*) mutant of *Drosophila* (Lin et al. 1998).

While there is good evidence for an age-related increase in oxidative damage of lipids, proteins and DNA, the relationship of mitochondrial oxygen radical generation or antioxidant capacity and chronological age seems not to exist. Instead, changes of the antioxidant capacity with age can vary between antioxidant and between tissues of an organism, and also depend on species specific lifestyle (Buttemer et al. 2010). Further, in transgenic animals, overexpression or knock out of the classical antioxidant enzymes such as MnSOD, Cu,ZnSOD, catalase or glutathione peroxidase (GPX) gave no consistent results in respect to life expectancy although in most cases animals over-expressing these genes showed a higher resistance to oxidative stress, whereas knock out animals were more vulnerable to oxidative stress (Jang et al. 2009; Pérez et al. 2009).

The correlation of ROS production in mitochondria and life span was investigated in diverse mammalian and avian species. Mitochondrial ROS generation was found to be lower in long-lived compared to short-lived species thus tending to inversely correlate with species lifespan (Barja 1999; Lambert et al. 2007). However, the existing data are still under debate. One argument is that most measurements on mitochondrial isolates were undertaken under normoxic (21kPa) conditions whereas the actual mitochondrial environment, the cytosol, normally is characterized by much lower oxygen concentration of about 1kPa. Thus these measurements might not show the true picture.

Aging in marine animals

Although studies of aging in marine vertebrates and invertebrates have more than 50 years of history (Haranghy et al. 1964; Woodhead 1974) compared to mammals, *Drosophila* and *Caenorhabditis,* they received far less attention until the recent decades (Reznick et al. 2004; Genade et al. 2005; Gerhard 2007; Philipp and Abele 2010).

As in all aerobic organisms, free oxygen radicals are produced in marine organisms in the course of cellular respiration. However, ROS generation may be significantly enhanced by exposure to various adverse environmental factors such as temperature extremes, hypoxia, UV radiation, xenobiotics, etc. In aquatic organisms the majority of studies investigating oxidative stress are focused on the effects of environmental factors (Viarengo et al. 1989; Viarengo et al. 1995; Buchner et al. 1996; Regoli et al. 1997; Estevez et al. 2002; Keller et al. 2004). Aging studies mostly concerned growth, reproductive potential and the identification of the chronological age, (MacDonald and Bayne 1993; Brey et al. 1995; Ziuganov et al. 2000; Cailliet et al. 2001; La Mesa and Vacchi 2001; Sukhotin and Flyachinskaya 2009). 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 (Zielinski and Pörtner 2000; Sukhotin and Pörtner 2001; Sukhotin et al. 2002; Correia et al. 2003; Heilmayer et al. 2003; Philipp et al. 2005b; Philipp et al. 2006; Ivanina et al. 2008; Philipp et al. 2008; Philipp and Abele 2010).

Aging and free radicals in fish

Fish represent the largest and very diverse class of vertebrates comprising more than 28,000 marine and freshwater species (Nelson 2006). The class displays a wide variety in life histories, reproductive strategies, age-related mortality rates and therefore rates of aging, and range in longevity from several months to more than 200 years (see Table 1). Some species such as the Pacific salmon, *Oncorhynchus* spp., eel, *Anguilla* spp., or capelin, *Mallotus villosus*, are semelparous with rapid aging and high mortality rates after their first spawning. Many species of teleost fish demonstrate slow albeit steady increase of mortality with age usually after reaching maturity when somatic growth becomes negligible (Woodhead 1979). Among them are the most popular piscine models for aging research – guppies, killifish and zebrafish. Finally, there are fish with extremely long life spans, associated with persistent slow growth over lifetime, and very low mortality rates at old age, in other words, showing negligible senescence. These include sturgeons *Acipenser* spp, and *Huso huso*, warty oreo *Allocyttus verrucosus* and rockfish *Sebastes* spp.

Historically fish were the first organisms with asymptotic ('infinite') growth involved in aging research, which lead G.P. Bidder (1932) to an erroneous conclusion about the lack of senescence and potential immortality of fish. He hypothesized that due to unlimited growth fish were able to constantly repair and replace damaged cells and tissues, whereby escaping senescence and achieving immortality. This concept has been thoroughly disproved by aging studies in the laboratory (e.g. Comfort 1960; Comfort 1961), as well as in wild fish populations (for review see Woodhead 1998).

Finch and Austad (2001) suggested two criteria for the absence of senescence: 1) no observable decline in reproduction rate or increased mortality after maturation and 2) no age-related decline in physiological performance or disease resistance. Like other vertebrates, fish do not meet these criteria and instead show features of aging across all studied species.

The use of fish as models in oxidative stress studies has started relatively recently (Kelly et al. 1998, this book sections 2.3, 4.2, 4.5). The accumulated data on age-related changes in lipid peroxidation in fish are still quite controversial. In larvae and fry of several fish species, the tissue concentrations of extractable FAPs decreased with age (Hammer 1988; Mullin and Brooks 1988; Hill and Womersley 1991; Mourente and Diaz-Salvago 1999). In brain of Dover sole *Microstomus pacificus*, an initial age-related increase of lipofuscin concentration reached a plateau after 15 years, whereas in the short-lived and fast-growing rainbow trout, *Salmo gairdneri*, brain and liver lipofuscin concentrations initially declined to stabilize in individuals older than 1 year (Vernet et al. 1988). Contrary, Passi et al. (2004) observed age-related exponential accumulation of lipid and protein oxidation products in adult rainbow trout and slower but significant accumulation of both substances in sea bass *Dicentrarchus labrax*. Morphological lipofuscin has been reported to accumulate in liver of annual killifish *Nothobranchius furzeri* (Genade et al. 2005), while in the other short-lived piscine gerontological model object, the zebrafish *Danio rerio,* no lipofuscin fluorescence could at all be detected (Kishi et al. 2003). Contrary, oxidized proteins accumulated in ske-

letal muscles of zebrafish over age. Zebrafish appear to undergo 'very gradual senescence' based on molecular changes including senescence-associated β-galactosidase activity and accumulation of oxidized protein with age in the face of continuously proliferating myocytes and constitutive telomerase activity in adult individuals (Kishi et al. 2003; Keller and Murtha 2004). The pattern of age-associated oxidative damage accumulation reflects the complex interplay of functional change in aging animals (e.g., metabolic alterations, fluctuations in antioxidant defenses or cellular repair mechanisms), including the level of pro-oxidants and their target molecules like polyunsaturated fatty acids, which are readily oxidized by ROS, or changes in environmental factors. Hence, a linear increase of lipofuscin concentration with age suggests a constant rate of ROS production, constant oxidative damage balanced by antioxidant protection, and constant aging rate. Acceleration of lipofuscin accumulation may indicate an increase of oxidative stress at advanced age possibly caused by age-related weakening of antioxidant defense. Slowing down of lipofuscin accumulation during aging may denote attenuation of oxidative stress by increased production of antioxidants or by metabolic decline at advanced age, or it could be the consequence of the 'dilution' of lipofuscin granules with age in fast growing young tissue/organism (see also Sukhotin et al. 2002). This phenomenon is well known from human fibroblast cell cultures (Sitte et al. 2001). Heat shock proteins (Hsps) play a key role in reparation, re-folding or elimination of damaged or denaturated proteins and could be important antagonists of aging. It is therefore interesting that aging modulated heat shock responsiveness and Hsp70 expression in zebrafish (*Danio rerio*) (Keller and Murtha 2004). Specifically, both basal and induced levels of Hsp70 were reduced in mature versus young zebrafish, indicating age-associated decline of repair capacity.

Aging and free radicals in bivalves

Bivalves were recently appreciated as potent models of aging. Some species are of extraordinary longevity >400 years, and the bivalve shell permits the determination of individual chronological age from annually forming age rings, at least in polar and temperate species (Fig. 1, see section below).

Bivalves belong to different eco-types which may influence the aging process. Infaunal bivalves burrow in the sediment and are usually of low metabolic rate and low scope for activity. These bivalves often show declining mitochondrial function (respiration, citrate synthase activity) as well as increased accumulation of cellular damage (protein carbonyls, malondialdehyde, lipofuscin) with age (Philipp et al. 2005b; Strahl et al. 2007; Abele et al. 2008). In contrast, pectinids or scallops are epibenthic (= living above the sediment), active swimmers with comparatively higher scope for activity and metabolic rate. In these animals mitochondrial function seems to be conserved, whereas mitochondrial volume density and citrate synthase activity diminish over lifetime (Philipp et al. 2006; Philipp et al. 2008).

Decline of some antioxidants (catalase, glutathione) and increase in oxidative damage with age was detected in some mussels, clams and scallops (Viarengo et al. 1991; Canesi and Viarengo 1997; Lomovasky et al. 2002; Sukhotin et al. 2002; Philipp et al. 2006; Philipp et al. 2008) as well

as in other mollusks such as gastropods and cephalopods (Clarke et al. 1990; Zielinski and Pörtner 2000) but cannot be generalized. Shorter lived species show faster increase in damage parameters and decrease in antioxidants compared to longer-lived species of similar lifestyle (Philipp et al. 2005a; Philipp et al. 2005b; Philipp et al. 2006). In the temperate clam *Mya arenaria* and the Antarctic clam *Laternula elliptica*, shorter lifespan correlated with higher mitochondrial ROS production in *M. arenaria*, where ROS formation accelerated with age. In contrast longer lived bivalve models apparently maintain mitochondrial ROS production low and stable over much of their lifetime (Philipp et al. 2005a). Similar as in fish, changes of cellular aging parameters are mostly non-linear. For *Mya arenaria* and *Mytilus edulis* an initial decline in lipofuscin at younger ages was followed by an increase at later age (Hole et al. 1995; Philipp et al. 2005b). In the long lived Iceland clam *Arctica islandica* which lives >400 years (Wanamaker et al. 2008), physiological aging appears to be close to negligible (Abele et al. 2008). The observed decline in antioxidants (catalase, glutathione) and the mitochondrial marker citrate synthase was observable only during the first 30 years of life, thereafter values remained constant for another 150 years of sampled lifespan. The early decline in antioxidant activities reflects physiological changes during maturation and not senescence. Oxidative damage parameters (lipofuscin, protein carbonyls) were very low compared to shorter-lived bivalves which appears essential for reaching long lifespan (Strahl et al. 2007).

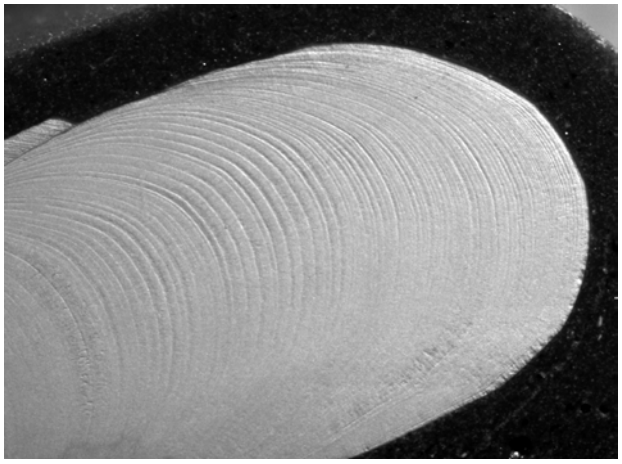

Fig. 1: Age rings in the umbo of the shell of Iceland *Arctica islandica*.

Age-related molecular changes in bivalves go hand in hand with shifts in cellular function and stress response over age. A swimming experiment with queen scallops demonstrated differences in the capacity for exercise swimming and stress response in young and old specimens. In young animals exhaustive swimming resulted in a faster decrease of muscle pH and glutathione levels as well as in a reduction of muscle glycogen compared to older scallops going through the same exercises. Young animals closed their shells more often and for longer times upon predator attack, whereas older animals kept the shells open, which matched their lower aerobic (mitochondrial volume density and citrate synthase activity) and anaerobic (glycogen concentration) energetic capacity (Philipp et al. 2008; Schmidt et al. 2008). Hypoxia and temperature experiments with *M. edulis* indicate that younger mussels may be more stress resistant than older individuals. Younger animals showed faster recuperation of lysosomal integrity destabilized by hypoxia and

hyperthermia than older individuals (Hole et al. 1995). In an anoxia reperfusion experiment older animals showed a marked increase in oxidative damage following reperfusion, whereas in younger animals lipidperoxidation markers remained on a low level (Viarengo et al. 1989).

The *Mytilus* studies must however be taken with a grain of salt, as in these studies age was deduced from size and not by individual age determination through shell rings counts. As shown in several studies by Sukhotin et al. (2001; 2002; 2003) size and age are not necessarily coupled in *Mytilus*, which is also the case in many other bivalve species, especially after reaching asymptotic growth, and the change with age in the investigated parameters can be quite different when plotted against age or size. Catalase for example decreased significantly when plotted against size in *M. edulis*, whereas no significant change was observed when data were related to age (Sukhotin et al. 2002). This example underlines the importance of the individual age determination in aging studies.

Aging in crustaceans

ROS related aging in crustaceans grew from two main standpoints: One relates from the practical need of separating age cohorts in population studies, especially of commercially important species. Crustaceans lack permanent calcified hard structures which could potentially bear age markers; therefore population age structure is usually determined through the analysis of size-frequency distribution which identifies modal size classes and considers them to reflect age-cohorts (Sheehy 2002; Harvey et al. 2008; Sheehy 2008). Although widely used, this procedure is rather imprecise. A second, alternative method for age estimation was proposed by Etterhank (1983; 1984). This method is based on the quantification of lipofuscin in post-mitotic cells (mainly nervous tissue). Lipofuscin accumulates as non-degradable end product of lipid peroxidation and protein damage due to oxidative stress. Since oxidative stress in animals depends on a combination of external and internal factors including metabolic activity, accumulated lipofuscin reflects ‘physiological’ rather than ‘chronological’ age (Bluhm et al. 2001). Studies so far demonstrated an age-related increase of lipofuscin or other FAP concentrations in tissues of crustaceans. The rate of FAP accumulation is however variant – it can be linear: lobsters *Hommarus gammarus, Panulirus cygnus*, and *P. argus* (O'Donovan and Tully 1996; Sheehy et al. 1998; Maxwell et al. 2007), crayfish *Cherax quadricarinatus* and *Pacifastacus leniusculus* (Sheehy 1996; Belchier et al. 1998), amphipod *Waldeckia obesa*, (Bluhm et al. 2001) and crab *Carcinus pagurus*, (Sheehy and Prior 2008), or accelerating with age in an exponential manner or as power function: crabs *Callinectes sapidus*, (Ju et al. 1999; Ju et al. 2001), lobster *Homarus americanus*, (Wahle et al. 1996) and shrimp *Aristeus antennatus*, (Mourente and Diaz-Salvago 1999), or decelerating with age: crayfish *Cherax quadricarinatus* (Sheehy 1992) and prawns *Penaeus japonicus* (Vila et al. 2000).

Trends in antioxidant activity and pro-oxidants levels (e.g., PUFA) in aging crustaceans are controversial. Thus activities of some antioxidant enzymes (catalase, glutathione transferase) were reported to decline, while others (SOD and GPx) feature an upward trend with age, e.g. in the shrimp *Aristeus antennatus* (Mourente and Diaz-Salvago 1999). In the planktonic cladoceran

Daphnia magna catalase, SOD and GPx activities mildly declined over age and significantly increased in the specimens of the oldest age group (Barata et al. 2005). However no difference in activities of the same enzymes could be observed between sub-adult and adult *Gammarus locusta* individuals, but were higher in juveniles of the amphipod (Correia et al. 2003). Overall, crustaceans clearly demonstrate progressive increase of oxidative damage with age, detectable as accumulation of lipofuscin and other fluorescent age pigments in cells.

Temperature and aging in marine animal ectotherms

Most cellular processes as well as almost all physiological rates, such as rates of respiration, feeding, growth and locomotion, strongly depend on body temperature, which in ectotherms depends on the environmental temperature. Also the complex biology of aging with its multiple dependence on metabolic rate is likely influenced by environmental temperature in aquatic ectotherms. Several comparative studies on wild populations of fish and invertebrates suggest that lifespan in congeneric species, at least in those with similar lifestyle, increases towards the poles (e.g. Ziuganov et al. 2000; Bluhm et al. 2001; Philipp et al. 2005b; Philipp et al. 2006; Sukhotin et al. 2007) and towards the deep ocean (Koslow et al. 2000; Cailliet et al. 2001). One of the hypotheses aimed at explaining these observations relates the prolonged lifespan with retardation of aging by the decelerating effect of low temperatures on ectotherm metabolic rate (e.g. Cailliet et al. 2001). Indeed, the first experimental confirmation of temperature effecting aging in aquatic vertebrates has been obtained by Walford and Liu (1965) studying small short-lived killifish *Cynolebias adolffi*. Rearing fish at lower temperature increased lifespan due to a fundamental change of the aging process. In fish maintained at low temperatures, morphological signs of aging such as decreased spinal curvature, eye cataracts, and scale alterations, as well as reproductive senescence were greatly delayed (review by Yen et al. 2004). Later, retardation of age-related collagen cross-linking was recorded in another species *Cynolebias bellottii* at low temperature (Walford et al. 1969), which served as an additional confirmation of the temperature effect on aging in fish.

The most widely accepted explanation of the temperature-related modulation of the aging rate lies within the framework of the metabolic and oxidative stress theories of aging. In ectotherms environmental temperature directly affects metabolic rate, and therefore the rate of ROS production. The destructive action of free radicals in cells is believed to be the primary cause of aging (Sohal 2002). Hence, the rate of aging is thought to be positively correlated with temperature through the metabolic rate. Valenzano et al. (2006) showed increased longevity in fish *Nothobranchius furzeri* reared at 22°C compared to fish reared at 25°C and a decreased accumulation of lipofuscin indicating the lowered levels of oxidative stress in cold acclimated fish. There is evidence, however, that aging retardation at low temperatures occurs due to mechanisms other than metabolic slow down although these mechanisms are not yet fully understood and require further investigation (Yen et al. 2004, and references therein).

Temperature effects on age-related oxidative damage determined as lipofuscin accumulation in crustaceans were extensively reviewed by Sheehy (2002). Generally, the rate of lipofuscin accu-

mulation in brain of the studied crustacean increased significantly with rising temperature. Thus, in a freshwater crayfish *Cherax quadricarinatus*, lipofuscin accumulated in a temperature-dependent manner with maximal rate at 28°C and minimal at 13°C (Sheehy et al. 1995). Exponential increase of lipofuscin levels at elevated rearing temperature was found also in juvenile lobster *Homarus gammarus* (O'Donovan and Tully 1996). The rate of lipofuscin accumulation and its relation to temperature can change with increasing age. In the crayfish *Cherax quadricarinatus* lipofuscin accumulation declined in older individuals at three different temperatures (Sheehy 1992) caused either by the age-related decline of metabolic rate or some kind of compensation or protection against oxidative damage activated at advanced age. The temperature dependence of lipofuscin accumulation rate is often non-linear with an increased sensitivity to temperature changes on climatic gradients. For example, according to the model of Sheehy (2002) an increase of only 0.5 °C in average annual sea water temperature from 10 to 10.5 °C would lead to more than a doubling of the rate of lipofuscin deposition and therefore greatly accelerated physiological aging in cold water populations of *Homarus gammarus* from N. Scotland. This highlights the fact that current patterns of global warming could lead to severe reductions in lifespan and age of maturation in many marine ectothermal populations.

An important indirect evidence of the temperature effect on aging in aquatic animals is provided in a number of publications on inter-specific comparisons of metabolism and aging in latitudinally separated bivalve mollusks of similar lifestyles (Philipp et al. 2005a; Philipp et al. 2005b; Philipp et al. 2006; Abele et al. 2009; Philipp and Abele 2010). Actively swimming scallops from temperate (*Aequipecten opercularis*) and Antarctic (*Adamussium colbecki*) regions differ significantly in their maximum lifespan from 8-10 years to >18 years, respectively. Oxidative stress was higher in the temperate short-lived species, reflected by more pronounced age-related lipofuscin accumulation and rapid decline in antioxidant activity compared to its cold adapted counterpart (Philipp et al. 2006). A comparison of two sessile infaunal clams – a cold-water stenotherm *Laternula elliptica* from Antarctica and a eurythermic temperate *Mya arenaria* - demonstrated fundamental differences in metabolic strategies that allow the Antarctic species to live 3-times longer than *Mya arenaria* (36 *vs* 13 years, respectively) (Philipp et al. 2005a; Philipp et al. 2005b). The Antarctic clam has lower whole animal metabolic rates, showed lower rates of mitochondrial H_2O_2 generation and maintained high glutathione levels compared to *M. arenaria*. Moreover, the long-lived *L. elliptica* was better able to conserve mitochondrial integrity and functional efficiency (i.e. high aerobic capacity, high respiratory control ratio) and featured a relatively high mitochondrial proton leak - the futile proton penetration pore across the inner mitochondrial membrane (Philipp et al. 2005a). Both, 'mild uncoupling' of mitochondria due to increased proton leak and high glutathione concentration theoretically inhibit ROS production in the Antarctic. Two hypotheses were derived concerning the prime cause of this strategy (Abele et al. 2009). Firstly, high proton leak and glutathione levels in *L. elliptica* tissues could be a mechanism of ROS reduction and possible life extension. This effect would be strictly species-specific and has nothing to do with cold adaptation. The second hypothesis is based on the fact that metabolic cold adaptation in animals often requires increased number of mitochondria or elevated inner mitochondrial membrane area (cristae density) to compensate for

low aerobic scope and deferred oxygen diffusion in the cold (Hochachka and Somero 2002; Pörtner 2002). Proton leak has been shown to positively correlate with the number of mitochondria, inner mitochondrial membrane area and the unsaturation ratio of mitochondrial membranes (Pörter et al. 1996). Therefore, increased cristae density and/or proton permeability of membranes obligatory increase proton leak, which in turn mitigates deleterious ROS production whereby prolonging lifespan of cold adapted animals. Thus, longevity may be a 'by-product' of metabolic cold adaptation and thus generally applicable to polar species.

Record holders of extremely short and long life spans in marine organisms

Extremely short and long lived multicellular animal species can be found in the aquatic ecosystem and are coming more and more into focus as model organisms for comparative aging research. One of the shortest-lived fish species is the coral reef pygmy gobi *Eviota sigillata* with an maximum lifespan of 56 days (Depczynski and Bellwood 2005). Other short lived examples are the freshwater killifish *Nothobranchius furzeri* and the transparent goby *Aphia minuta* (Caputo et al. 2002). *N. furzeri* has a maximum lifespan of only 13 weeks and lives in the dry Lowveld of Zimbabwe. It can survive the long dry season (>10 months) as embryos encased in the dry mud (Valdesalici and Cellerino 2003). This species shows explosive growth, early sexual maturation and age-dependent physiological decline, and expresses age-related biomarkers such as lipofuscin and β-galactosidase (Terzibasi et al. 2007). Current efforts are under way to use this species as a new vertebrate model of aging, and first genome sequences have been obtained (Terzibasi et al. 2007; Reichwald et al. 2009). The pelagic planktophagous marine goby *A. minuta* also lives <1 year. In this species programmed cell death (apoptosis) leads to immediate death after breeding, hence the fish is used as a model to understand general mechanisms of apoptosis and its involvement in the aging process (Caputo et al. 2002). On the other hand of the age spectrum, the group of fish includes super-centenarians, such as the most long-lived vertebrate species with testified lifespan of 205 years – the rockfish *Sebastes aleutianus* (Cailliet et al. 2001).

Another group with very short but also very long-lived species are the bivalve mollusks. The species *Pisidium* spp, *Donax* spp. and *Argopecten irradians irradians* live only 1-2 years, whereas the bivalve *Arctica islandica* probably is the longest-lived non-colonial animal on earth. Around Iceland individuals of this species have extremely long life spans of >400 years (Table 1) with slow rates of cellular senescence (Abele et al. 2008; Wanamaker et al. 2008). Different populations of *A. islandica* with different maximum lifespan from diverse marine environments in the northern hemisphere are known. Therefore this species represents a valuable model to study the evolution of lifespan in different climates and to investigate how longevity is shaped under different environmental and genetic settings.

Technical section: age estimation in marine organisms

Reliable age determination methods form the basis of investigations of animal growth, development and physiological aging. They are also essential for ecological management of commercially important marine species, as they enable researchers to determine life time growth history, maximum life spans, and the age of reaching sexual maturity in a given species. Further, physiological parameters such as metabolic rate, antioxidant defense and the accumulation of oxidative damage can be related to chronological age to describe the dynamic process of aging in long and short-lived marine ectotherms (Zielinski and Pörtner 2000; Sheehy 2002; Kishi 2004; Passi et al. 2004; Abele et al. 2008; Philipp et al. 2008; Philipp and Abele 2010).

Age estimation using hard structures

In aquatic animals, periodic growth increments of hard structures are often used to estimate age and growth rates, such as teeth of elephant seals and dolphins (Laws 1952; Hohn 1980), bones of turtles (Zug et al. 1986; Parham and Zug 1997), vertebrae, scales and otoliths of fish (Chilton and Beamish 1982; Penttila and Dery 1988; Cailliet et al. 2001; Yilmazy and Polat 2002), brittle star skeletons (Gage 1990), statoliths in squids or cubomedusae (Ueno et al. 1995; Arkhipkin 1997), coral skeletons (Dodge and Thomson 1974) and bivalve shells (Hendelberg 1960; Heilmayer et al. 2003; Schöne 2003; Begum et al. 2009). All these organisms exhibit growth band patterns of different density or structure, triggered either by environmental factors such as changes in temperature and food supply, or by internal factors such as reproduction.

The most common method of age determination in fish is to count the age rings on the otolith cross-sections (Chilton and Beamish 1982; Christensen 1984). In bivalves growth lines of younger individuals are mostly visible on the external shell surface, but age determination of older animals has to be conducted on cross sections of the shells. The surface of the shell cross sections is ground and polished to visualize age rings, which can be counted either along the outer shell line or inside the umbo/hinge using a stereomicroscope (Fig. 1). Thereby, growth increments appear as wide and light bands of homogenous structure and the growth lines as narrow and irregular dark rings (Jones 1980; Ropes et al. 1984). The growth ring pattern is however not necessarily formed at regular intervals in time, and more than one growth increment per year can be deposited. Disturbance rings can be produced due to environmental conditions, such as salinity or temperature changes, or to reproductive events.

To determine whether growth layers are formed annually or how many layers are built per year or day, age determination methods using otoliths, bivalve shells or other hard structures like coral skeletons have to be validated. Tag-recapture or radiometric approaches based on known radioactive decay series are useful to validate the assumed annual growth band formation in fish otoliths and bivalve shells. The analysis of different isotope pairs from the uranium and thorium decay

Species	MLSP (years)
Yeast (*Saccharomyces cerevisiae*)	0.04
Nematoda (*Caenorhabditis elegans*)	0.16
Turquoise killifish (*Nothobranchius furzeri*)*	0.25
Transparent goby *(Aphia minuta)**	1
Fruit fly (*Drosophila melanogaster*)	0.3
Bay scallop (*Argopecten irradians*)*	1-2
Honey bee (*Apis mellifera*)	8
Queen scallop (*Aequipecten opercularis*)*	8-10
Antarctic mud clam (*Laternula elliptica*)*	36
Gray seal *(Halichoerus grypus)*	43
Herring gull (*Larus argenatus*)	49
Manx shearwater *(Puffinus puffinus)**	50
Gorilla (*Gorilla gorilla*)	55
Royal albatross (D*iomedea epomophora)**	58
Common limpet (*Nacella concinna*)*	> 60
Black-headed gull (*Larus ridibundus)**	63
Raven *(Corvus corax)*	69
Splitnose rockfish (*Sebastes diploproa*)*	80
American lobster (*Homarus americanus*)*	100
Deep-see clam (*Tindaria callistiformis*)*	> 100
Bowhead whale (*Balaena mysticetus*)	> 100
Human (*Homo sapiens*)	123
Warty oreo (*Allocyttus verrucosus*)*	140
Orange roughy (*Hoplostethus atlanticus*)*	149
Lake sturgeon (*Acipenser fulvescens*)*	152
Geodruck clam (*Panopea abrupta*)*	163
Tortoise (*Geochelone elephantopus)**	177
Freshwater pearl shell (*Margaritifera margaritifera*)*	190
Red sea urchin (*Strongylocentrotus fransciscanus*)*	200
Rockfish (*Sebastes aleutianus*)*	205
Ocean quahog (*Arctica islandica*)*	407
Epibenthic sponge (*Cinachyra antarctica*)*	1550
Hexactinellid sponge (*Scolymastra joubini*)*	15 000
Hydra (*Hydra* spp.)*	non-aging?

Table 1: Maximum life span (MLSP) of different animals throughout the animal kingdom. Bivalve life span range is 1-407 years, fish life span range is 0.25-205 years, and the oldest animals known so far are sponges with maximum ages up to several thousand years. Life spans data sources: for *Argopecten irradians* (Estabrooks 2007), *Nacella concinna* (Picken 1980), *Sebastes diploproa* (Bennett et al. 1982), *Tindaria callistiformis* (Turekian et al. 1975), *Balaena mysticetus* (George et al. 1999), *Hoplostethus atlanticus* (Fenton et al. 1991), *Panopea abrupt* (Strom et al. 2004), *Margaritifera margaritifera* (Ziuganov et al. 2000), *Arctica islandica* (Wanamaker et al. 2008), Hydra (Martinez 1998); other life spans are from AnAge database. * aquatic ectothermal species.

series has been used in aging studies with each isotope pair being applied in a corresponding time range. Thus, ^{210}Po/^{210}Pb is used in the range 0-2 years in *Nautilus* (Cochran et al. 1981), ^{228}Th/^{228}Ra is applied in the 0-10 years range in corals and clams (Dodge and Thomson 1974; Turekian et al. 1975), while ^{210}Pb/^{226}Ra is used in the range 0-100 years in fishes (Fenton et al. 1991).

Isotope profiles of shell material taken over the growth line also give further information on present-day and former environmental conditions (Turekian et al. 1975; Brey and Mackensen 1997; Schöne et al. 2004). Bivalve shells are thus increasingly used not only for individual age determination, but also as palaeoclimate archives (Schöne et al. 2004; Schöne et al. 2005). Analogous to trees, growth rings of the long-lived bivalve *Arctica islandica* are investigated to establish climate models covering the last hundreds of years (Schöne et al. 2005). In each growth increment, the ratio of oxygen ^{18}O and ^{16}O or carbon ^{13}C and ^{12}C isotopes mirrors environmental factors such as temperature and the content of seawater carbonate during the time of increment formation (Brey and Mackensen 1997).

Growth increments have also been found in skeletal structures of deep-sea brittle stars as fine, translucent rings, separated by wider, more opaque zones in the calcitic arm ossicles (Gage 1990), in statoliths of squids and jellyfish (Ueno et al. 1995; Arkhipkin 1997), or even in bones of amphibians and sea turtles (Smirina 1994; Parham and Zug 1997). But as these increments are predominantly assumed to form annually in these animal groups, without an ample validation of data sets, age determinations have to be regarded with caution. In contrast, coral growth rings, visible as bands of variable skeletal density in the growth direction of the corals, are annually formed. Corals from Eniwetok in the Pacific Ocean had radioactive nucleotides incorporated from US fission device testing in the years from 1948 to 1958, and the radioactive marker layers were in agreement with visible annual growth rings (Knutson et al. 1972).

In marine mammals different age determination methods exist, including the count of growth layer groups in teeth, which are considered to form annually in many odontocetes (Hohn 1980), or the analysis of tissues in mysticetes, which form horny growth layers called ear plugs in the external auditory meatus (Slijper 1962), or the aspartic acid racemization technique in the eye lens of bowhead whales (George et al. 1999) which measures the D/L ratio of the two isomeric forms of the aspartic acid (Bada et al. 1980).

Biochemical and physiological proxies of aging

Alternative methods for age determination in animals lacking permanent hard structures are based on biochemical or physiological proxies in cells and tissues, such as concentrations of the age pigment lipofuscin (see this chapter, above). Studies on crustaceans and bivalves show a clear relationship between age and lipofuscin concentration, and support the idea that lipofuscin is a suitable marker for both physiological and chronological age (Bluhm et al. 2001; Lomovasky et al. 2002; Sheehy 2002). However, lipofuscin as an age marker has to be calibrated for each species

or population, as age pigment formation can be strongly affected by temperature or heavy metal contamination (see above). Telomere length is also discussed as a proxy for age. Telomeres are highly conserved repetitive DNA-sequences and associated proteins that cap the end of eukaryotic chromosomes, and provide chromosomal stability or promote replication processes and chromosome segregation. In many vertebrate species telomeres shorten during each cell division cycle, because the DNA polymerase is unable to replicate the ends of DNA molecules. At a critical length of the telomeres, cell division stops and replicative senescence starts. The enzyme telomerase can maintain or even re-elongate telomeres through telomeric DNA synthesis. In several aquatic vertebrate and invertebrate species the relationship between telomere length or telomerase activity and individual age was analyzed, to test whether or not telomere length could be used as a reliable marker for age determination. But the picture is not consistent across species and not even between different tissues in one species. A positive correlation of telomere length with lifespan was found for short-lived and long-lived scallop species (Estabrooks 2007). In contrast, telomere length in two long-lived birds both increased and declined throughout life (Hall et al. 2004). Lack of age-associated telomere shortening was reported for zebrafish (Lund et al. 2009) and for short- and long-lived sea urchin *Lytechinus variegates* and *Strongylocentrotus franciscanus* (Francis et al. 2006). Therefore, telomere length cannot be considered a reliable age marker.

In summary common biochemical or physiological proxies for age are difficult to find in marine animals, whereas periodic growth increments and isotope incorporation in hard structures represent an applicable tool for age determination in many species.

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5. ADDITIONAL RESULTS

In the following section I will present additional results from my laboratory work, not included in publications I - VI. Some experiments in this section still have to be repeated for final confirmation of the results.

5.1 Is nitric oxide a regulator of metabolic rate depression in the bivalve *Arctica islandica*?

This section comprises data on the inhibition of respiration in isolated gills of the clams by chemically introduced nitric oxide (NO formed from SpermineNONOate), and on the accumulation of nitrate and nitrite in different metabolic states of the ocean quahog *A. islandica* (normoxic versus metabolically depressed). Furthermore, preliminary data are shown on NO-generation by hemocyte cells of *A. islandica,* which, until now, could be measured only in one occasion because of time limited access to the ISO-NOP-electrode (ISO-NOP, World Precision Instruments, USA). These measurements of NO-production by hemocytes will be repeated in experiments starting in May 2011 at the AWI, using the ISO-NOP-electrode, which was bought for the laboratory of PD Dr. Doris Abele. As soon as I can finalize the measurements of NO-generation by hemocyte cells of *A. islandica*, the whole data-set shown in section 5.1 will be submitted for publication. Therefore, section 5.1 is already presented in the classical manuscript format.

5.2. Hemocyte cell cultures

This section represents part of my work in which I optimized primary cell culture conditions for hemocyte cells harvested from the ocean quahog *Arctica islandica* as a prerequisite for experimental work.

5.1. Possible functions of the signaling molecule nitric oxide in the bivalve *Arctica islandica* (preliminary chapter)

Introduction

The free radical nitric oxide (NO) is an important intra- and extracellular signalling molecule, and as such evolutionary and functionally conserved in invertebrates (Palumbo 2005). NO has various biological roles in the nervous, cardiocirculatory and immune system of marine invertebrates such as porifera, cnidaria, arthropoda, molluska, echinodermata and chordata, which are related to feeding, defense, environmental stress, learning, metamorphosis, swimming, symbiosis, as well as hemocyte aggregation and regulation of blood pressure (Palumbo 2005). In mollusks such as *Mytilus edulis* and the carpet shell clam *Ruditapes decussates*, NO produced by hemocytes leads to bacterial clumping and killing (Stefano and Ottaviani 2002) and to killing of invading pathogens (Tafalla et al. 2003). Furthermore NO is a potent messenger molecule in molluskan nervous systems (Moroz and Gilette 1995).

NO can be generated enzymatically by cytosolic (and presently unconfirmed in marine invertebrates: mitochondrial) nitric oxide synthase (NOS, Moroz and Gilette 1995) or by xanthine-oxidoreductase (Millar et al. 1998; Gladwin et al. 2005). NOS-like activity is reported for marine, freshwater and terrestrial mollusks (Jacklet 1997), i.e. in the digestive gland of *Mya arenaria* (González et al. 2008), in hemocytes of the freshwater snail *Viviparus ater* (Conte and Ottaviani 1995) and in the central nervous system (CNS) of the snail *Pleurobranchea californica* (Moroz et al. 1996). Marine invertebrate NOSs show putative cofactor recognition sites for heme, tetrahydrobiopterin, calmodulin, FMN, FAD and NADPH to catalyze the conversion of L-arginine to citrulline and NO, and seems to be related to the three major mammalian-NOS-isoforms: (1) the neuronal Ca^{2+}/calmodulin-dependent cytosolic enzyme, (2) the inducible, Ca^{2+}-independent membrane-associated and (3) the Ca^{2+}-dependent membrane-associated isoform (Palumbo 2005).

Non-enzymatically, NO is produced by acidic reduction of nitrite in vertebrate tissues (reductive sequence: $NO_2^- + H^+ \leftrightarrow HNO_2$ / $2\ HNO_2 \leftrightarrow H_2O + N_2O_3$ / $N_2O_3 \leftrightarrow NO + NO_2$) (Benjamin et al. 1994). The reductive process is favored at low pH and low oxygen tension and also occurs in anoxia (Zweier et al. 1999; Schreiber 2009). Furthermore NO can be generated in marine sediments and pore-water as intermediate or by-product during the microbial N-cycle under normoxic, hypoxic and anoxic conditions, i.e. during denitrification, the dissimilatory nitrate (NO_3^-) reduction to ammonium (NH_4^+) or during the anaerobic oxidation of NH_4^+ to N_2 with NO_2^- as electron acceptor (Strous et al. 1999).

NO is an uncharged molecule which passes freely through cellular membranes with a diffusion velocity of 3300 $\mu m^2\ s^{-1}$ (Malinski et al. 1993) and a maximum diffusion distance of the order of 100-200 µm (Lancaster 1997), allowing rapid diffusive distribution in an organism. The half-life of

NO in aqueous solutions is reported to be in the range of 5-15 s (Lancaster 1997) and in molluskan hemolymph it is significantly higher at physiologically low intracellular PO_2 of 1-5 kPa than at normoxic conditions of 20 kPa (Okada et al. 1996). The more oxygen is dissolved in an aqueous solution, the more rapidly NO autoxidizes to NO_2^- according to the reaction: $4NO + O_2 + 2\ H_2O \rightarrow 4\ NO_2^- + 4\ H^+$ (Pogrebnaya et al. 1975). When starting the project we conjectured that lowering the oxygenation level in molluskan hemolymph may support longer NO lifetime and higher NO concentration in the hemolymph. Moroz and Gillette (1995) report NO concentrations in molluskan CNS between 30 and 300 nM, and they attribute these to the rather low PO_2 they measured in molluskan hemolymph: 20-40 torr (2.5-5 kPa) in non-specified marine mollusks and 5-12 torr (0.7-1.6 kPa) in fresh water snails. Marine pectinids, mud clams and limpets were found to actively down-regulate mantle cavity water PO_2 also in a fully oxygenated environment (21 kPa), and in *Arctica islandica* from the German Bight and Kiel Bight mean mantle cavity water PO_2 was < 5 kPa (Abele et al. 2010; Strahl et al. in revision). Furthermore, *A. islandica* frequently closes their shell or burrows several into the sediment for time periods of 1-7 days, leading to a gradual decline in mantle cavity water PO_2 to 0 kPa and to a self-induced metabolic rate depression (MRD) (Taylor 1976; Strahl et al. in revision).

In vertebrate cells, NO has been recognized as a potent mitochondrial regulatory metabolite by directly inhibiting cytochrome-*c*-oxidase, the terminal electron acceptor of the mitochondrial electron transport chain (Boveris et al. 2000; Turrens 2003). Cytochrome-*c*-oxidase can be inhibited both in the oxidized and reduced state (Cooper et al. 1992). NO binding to the enzyme is reversible and competitive with oxygen and, therefore, depends on the cellular oxygen concentration (Boveris et al. 2000). High enough NO concentration causes complete inhibition of whole mitochondrial respiration (Cooper 2002). Thus NO may play a prospective role in the self-induced metabolic rate depression (MRD) in *A. islandica*, as prolonged burrowing or shell-closure lead to a decline in mantle cavity water and hemolymph PO_2 and, in so doing, increase the concentration of NO in the hemolymph. NO could then diffuse into the cells of tissues bathed in hemolymph to reduce mitochondrial respiration by inhibition of cytochrome-*c*-oxidase.

The aim of the present study was to measure formation of NO in hemolymph of the ocean quahog and to characterize the inhibition of NO on *A. islandica* tissue respiration. We indirectly determined NO formation by measuring nitrite and nitrate content in the hemolymph of bivalves incubated for 3.5 days under normoxic (21 kPa), hypoxic (2 kPa) or anoxic conditions (0kPa) as well as in the mantle cavity water of bivalves which were burrowed for 3.5 days in minimum 3 cm sediment depth. To confirm NO production by NOS, we measured NO formation with an NO-electrode in hemolymph of *A. islandica* with and without adding L-arginine. We hypothesized that NO will inhibit mitochondrial respiration in the clams, and that the reduction in respiration may differ at different PO_2 (normoxia → hypoxia) levels. Therefore, the effect of chemically produced NO on gill respiration rates was measured at normoxia (21 kPa), at stepwise reduced PO_2 (16 kPa, 10 kPa, 5 kPa), and at hypoxia (2 kPa).

Material and Methods

Bivalve collection and maintenance

Arctica islandica were collected in May 2008 at Helgoland 'Tiefe Rinne' in the German Bight (54°09.05'N, 07°52.06'E, Fig. 1, 2) at 40 − 45 m water depth, using a trawl net. Surface water temperature was 12°C. In August 2008, *A. islandica* were collected northeast of Iceland (66°01.44'N, 14°50.91'W, Fig. 1, 2) at 8–15 m water depth at a surface water temperature of 9°C. Bivalves from the German Bight and Iceland were transported in cooled containers to the Alfred Wegener Institute for Polar and Marine Research in Bremerhaven. *Arctica islandica* were acclimated for two weeks at 10°C and 33 PSU in 60-L tank with re-circulating seawater containing 10 cm of pea gravel sediment (2-3 mm grain size). Bivalves were fed once a week with a mixture of *Nannochloropsis occulato*, *Phaeodactylum tricornutum* and *Chlorella sp*. (DT´s plankton farm, USA, 3 ml bivalve^{-1} wk^{-1}).

Measurement of nitric oxide in hemolymph using an NO-electrode

In order to take 4 ml of mantle cavity water with a sterile needle and a sterile 10-ml-syringe, a 2 mm hole was drilled into the shell of *A. islandica* specimens from the German Bight about 1 cm from the shell edge.. The sample was centrifuged 10 min at 450 g and 10°C. The supernatant was then discarded and the pellet containing the hemocytes was diluted in 2 ml of filtered seawater. The NO-electrode (ISO-NOP, World Precision Instruments, USA) was inserted into the sample solution to continuously measure NO concentrations at low oxygen conditions (≈10 kPa). The sensor of the NO-electrode is amperometric, NO diffuses rapidly through the electrode's selective membrane and is oxidized at the 'working' electrode, resulting in the generation of an electrical (redox) current. The intensity of the current signal is proportional to the concentration of NO in the sample solution. NO concentration in the hemocyte solution was determined without arginine, and, in order to measure NO produced by NOS, after adding in two-minutes-intervals 20 µl, 100 µl and 500 µl of a saturated arginine-solution to the sample.

Incubation of A. islandica at three different PO_2

Arctica islandica from the German Bight were exposed individually in 3-L flasks filled with natural seawater at constant water temperatures of 10°C and salinity of 33 PSU as described in Strahl et al. (2011). Three days before experimental start, bivalves were not fed anymore to reduce eutrophication of the experimental water through faeces and microbial contamination. Specimens were incubated under normoxic (21 kPa), hypoxic (2 kPa) or anoxic (0 kPa) conditions using a gas mixture of nitrogen and oxygen (Air Liquide, Germany). Siphon status of each individual was visually checked and recorded three times per day during incubations. After 3.5 days of incubation, shell closure of each bivalve was prevented by inserting a metal bar (3 mm thick and 3 cm long). Hemolymph was taken from the adductor muscle of each individual with a sterile needle and a

10 ml-syringe, and nitrite and nitrate concentrations were determined individually in the hemolymph and in the incubation water of each bivalve with the Griess Assay.

Burrowing experiments of A. islandica

Wire straps of 18 cm length and 0.3 cm diameter were numbered and attached to shells of German Bight specimens next to the siphon openings with epoxy adhesive glue (Reef construct, Aqua medic, Bissendorf, Germany), which solidified completely underwater within 24 h. Clams were kept in 60-l tank containing 20 cm of pea gravel sediment. Daily burrowing depth of each clam was determined by measuring the length of the wire which was visible above the sand as described in Strahl et al. (submitted). Individuals which were burrowed for 3.5 days in more than 3 cm sediment depth were sampled as MRD-clams and bivalves that showed their siphons for 3.5 days at the sediment surface were sampled as normoxic-clams (= controls). A 2 mm hole was drilled into the shell of each bivalve about 1 cm from the edge to take mantle cavity water with a sterile needle and a 10-ml syringe. Nitrite and nitrate concentrations were determined with the Griess Assay in the mantle cavity water of four bivalves in the normoxic and MRD state, respectively, and in the aquarium water.

Measurement of nitrite and nitrate

Nitrite (NO_2^-) and nitrate (NO_3^-) content in aquarium water, mantle cavity water and hemolymph were measured with the Griess Assay according to Misko et al. (1993) and Verdon et al. (1994). Each sample was measured with and without the conversion of nitrate to nitrite to determine the overall concentration of nitrite + nitrate (∑NiNa) and only nitrite. To convert nitrate to nitrite 50 µl of sample, standards (standard 1: 125 µM nitrite, standard 2: 125 µM nitrate) or blank solution (miliQ-water) was mixed with 10 µl NADPH and 40 µl of freshly prepared master mixture (final concentrations: 1 µM NADPH, 500 µM glucose-6-phosphate, 160 U/L glucose-6-phosphat dehydrogenase, 80 U/L nitrate reductase and 14 mM sodium phosphate buffer, pH 7.2) and incubated for 45 min at 20°C. The efficiency of nitrate reductase to convert nitrate to nitrite was tested using a defined nitrate quantity (125 µM) and measuring of the resulting nitrite concentration. 100 µl of sample, blank or nitrate-standard (30 µl of 125 µM nitrate standard filled up with 70 µl of 14 µM sodium phosphate buffer) was mixed with 100 µl of each 1 % (v/w) sulfanilamide in 5 % phosphoric acid (SA) and 0.1 % (v/w) N-(1-naphthyl)ethylenediamine HCL (NED) (ratio 1:1:1). In order to measure only the nitrite content, in parallel each sample was mixed with SA and NED at the ratio 1:1:1 without pre-incubation. For the standard curve 30 µl (12.5 µM), 15 µl (6.3 µM), 10 µl (4.2 µM), 5 µl (2.0 µM) and 1 µl (0.42 µM) of the 250 µM nitrite standard was diluted to a volume of 100 µl with 70 µl, 85 µl, 90 µl, 95 µl and 99 µl of 14 µM sodium phosphate buffer and mixed with 100 µl SA and 100 µl NED, respectively. Samples, blank and standards were incubated for 10 min at 20°C to determine the nitrite content at 540 nm in a microplate reader (Sunrise, Tecan, Germany). Nitrite concentrations in the samples were calculated by using the standard curve after subtracting the value for the enzyme blank.

Measurement and NO inhibition of gill respiration at four different PO_2s

Bivalves were kept without food for three days before the experiment to eliminate the impact of specific dynamic action (SDA) on gill respiration (Bayne et al. 1976). Gills of Iceland *A. islandica* were freshly dissected and transferred to cooled respiration buffer (450 mM NaCl, 10 mM KCl, 20 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 0.5 mM DTT, 0.055 mM glucose, pH 7.4). Gills of each test animal were cut into two pieces of 15-25 mg wet mass (WM) and incubated for 2 h in respiration buffer at 8°C and a PO_2 of 2 kPa, 6 kPa, 10 kPa or 16 kPa, obtained by using a gas mixing pump (Wösthoff GmbH, Bochum, Germany). After incubation, one piece of gill from each individual was transferred into a thermostated respiration chamber filled with 1.1 ml respiration buffer at 8°C and the respective test PO_2. As described in Strahl et al. (2011), oxygen consumption rates of each gill piece was measured for 30 min at 2 kPa, 6 kPa, 10 kPa or 16 kPa, respectively, using single channel Microx TX-3 oxygen meters equipped with oxygen needle-optodes (PSt1-L5-TF, Precision Sensing GmbH, Germany), which were primarily calibrated to 100 % and 0 % O_2. The second gill piece of each specimen was measured in parallel in another chamber at 8°C. In this chamber gill respiration was inhibited with sperminNONOate at the respective test PO_2. When the correct PO_2 had established, sperminNONOate was injected into the respiration chamber where it releases NO. After determining the minimal necessary concentration of sperminNONOate to completely inhibit respiration for 20 min at each specific PO_2 level, we neutralized inhibition by adding a defined amount of the NO scavenger oxyhemoglobin and again determining the minimal necessary concentration. WM of each gill piece was determined and respiration rates were calculated as nmol O_2 min^{-1} ml^{-1} mg^{-1}WM. NO concentrations in the respiration chambers were calculated from known NO generation rates of specific sperminNONOate amounts, measured in seawater with an ISO-NOP-electrode at 2 kPa and 10°C (= 6 mM spermineNONOate generated 45 nM NO min^{-1}).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 Software (La Jolla, California,USA). All data sets were tested for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Bartlett's test) before testing for PO_2 specific differences in nitrite and nitrate content or gill respiration.

Preliminary Results

NO was produced by *A. islandica* hemocyte cells. The NO production of the hemocytes could be triggered by arginine, and the NO concentration in the sample increased in proportion to the added amount of arginine (Table 1).

All animals survived 3.5 days of experimental exposure to hypoxia and anoxia. We observed their siphons to be permanently open during hypoxic and anoxic incubations, whereas bivalves exposed to fully normoxic conditions alternately opened and closed siphons. In incubation water and

hemolymph of bivalves incubated 3.5 days under normoxia, hypoxia or anoxia, PO_2-dependent differences were detected in the nitrite, as well as in the ∑NiNa concentration (Fig. 1A, B).

Table 1: Nitric oxide (NO) production by hemocytes of German Bight *Arctica islandica*, measured as NO concentration in the hemocyte solution at 2 min intervals. HC = hemocytes in filtered seawater, AS = saturated arginine-solution, *n* = 1.

	NO (nM)	Time (min)
HC	6,2	2
HC + 20 µl Arg	1,7	4
HC + 100 µl Arg	13,7	6
HC + 500 µl Arg	22,4	8

The ∑NiNa content of incubation water and of hemolymph was significantly lower under hypoxic compared to normoxic conditions (Fig. 1A). Under anoxia the ∑NiNa content of the incubation water was two times lower than under hypoxic conditions and three times lower than under normoxic conditions (Fig. 1A). But in the hemolymph ∑NiNa was significantly increased under anoxia compared to hypoxia, and significantly higher than in the anoxic incubation water (Fig. 1A). Also the nitrite content alone in the anoxic hemolymph was significantly higher compared to normoxia and hypoxia as well as compared to the anoxic incubation water (Fig. 1A, B). The nitrite proportion of ∑NiNa in the hemolymph increased dramatically from 12 % under normoxia and 18 % under hypoxia to 98 % under anoxia (Fig. 1A, B). The nitrite content alone was similarly high under normoxic and hypoxic conditions in the seawater and hemolymph, respectively. Although the content of both∑NiNa and nitrite was always higher in hemolymph than in the incubation water under normoxia and hypoxia, differences were not statistically significant (Fig. 1B).

In the burrowing experiments the nitrite content in the mantle cavity water of MRD-clams was significantly higher compared to normoxic-clams and compared to the aquarium water (Table 2). The ∑NiNa content was similarly high in the aquarium water and in the mantle cavity water of normox- and MRD-clams (Table 2). All samples (mantle cavity water and aquarium water) of the burrowing experiment had > 10-times higher ∑NiNa contents compared to the incubation water and the hemolymph of bivalves incubated under normoxic, hypoxic and anoxic PO_2 (Fig. 1 and Table 2).

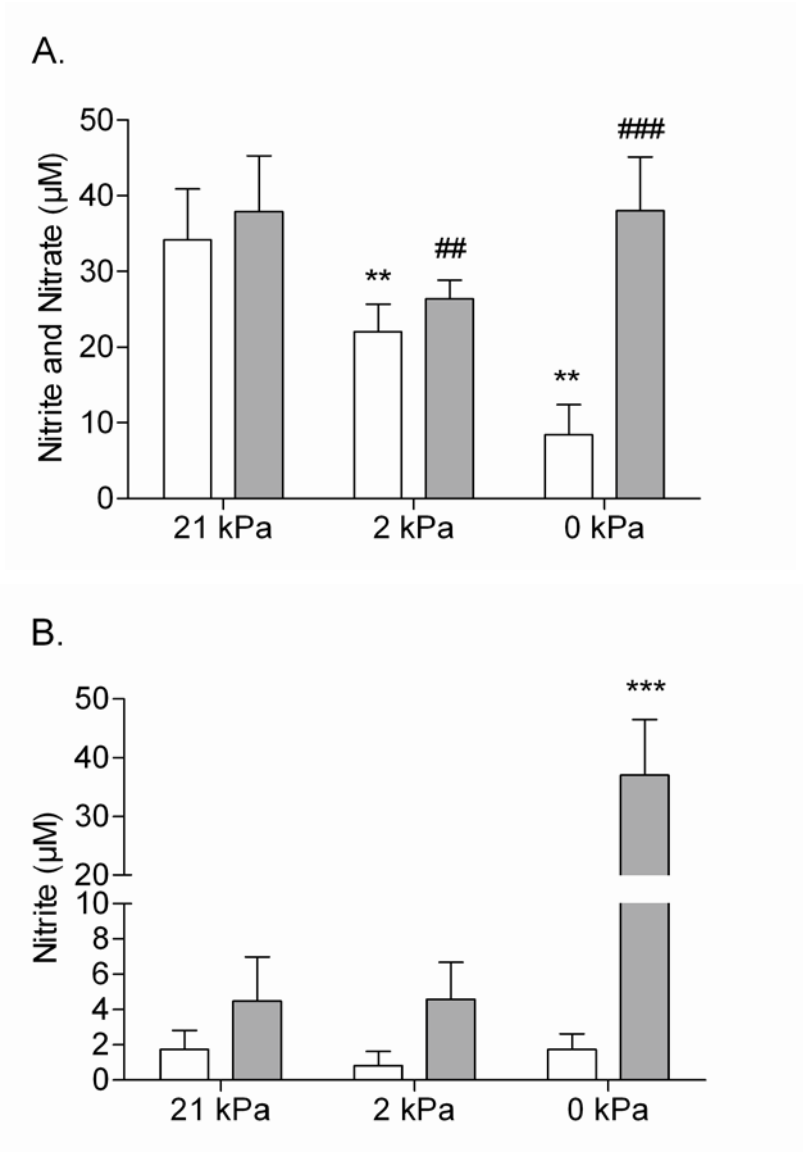

Fig. 1: Concentration of (A) nitrite and nitrate and (B) only nitrite in the incubation water (white bars) and hemolymph (grey bars) of German Bight *Arctica islandica* after 3.5 days of normoxic (21 kPa), hypoxic (2 kPa) or anoxic (0 kPa) incubation, mean ± SD, *n* = 6-10. ** Incubation water differs significantly between normoxic vs. hypoxic and anoxic conditions (One-way ANOVA $p < 0.0001$, Tukey $P < 0.01$). ## Hemolymph differs significantly between hypoxic vs. normoxic and anoxic conditions (One-way ANOVA $P < 0.0001$, Tukey $p < 0.01$). ### Under anoxic conditions hemolymph differs significantly from incubation water. *** Hemolymph differs significantly between anoxic vs. normoxic and hypoxic conditions, and anoxic hemolymph differs significantly from anoxic incubation water (One-way ANOVA $P < 0.0001$, Tukey $P < 0.001$).

Table 2: Concentration of nitrite and nitrate (∑NiNa) and only nitrite in the aquarium water and in the mantle cavity water of German Bight *Arctica islandica* after 3.5 days in the non-burrowed (normoxic-clams) or burrowed state (MRD-clams), mean ± SD, *n* = 3-4. ***Nitrite content in mantle cavity water of MRD-clams differs significantly compared to normoxic-clams and to aquarium water (One-way ANOVA P < 0.0001, Tukey P < 0.001).

	∑NiNa	Nitrite	% Nitrite of ∑NiNa
Aquarium water	627.6 ± 60.6	2.7 ± 1.2	0.4
Normoxic-clams	639.8 ± 61.5	6.0 ± 1.8	0.9
MRD-clams	554.3 ± 37.9	140.7 ± 20.2 ***	25.4

Gill respiration rates of Iceland *A. islandica* decreased with declining PO_2 in the incubation medium between 16 kPa and 2 kPa, but differences were not statistically significant (Table 3A). At 10 kPa mean gill respiration was 8 % lower, at 6 kPa 36 % lower and at 2 kPa 85 % lower compared to oxygen consumption rates at 16 kPa (Table 3A). Gill respiration was completely inhibited by different NO concentrations at 2 kPa, 6 kPa and 10 kPa (Table 3A) after adding a defined amount of sperminNONONate (see minimum needed sperminNONOate concentrations for inhibition in Table 3B). At a PO_2 of 16 kPa, gill respiration was reduced after adding the fivefold amount of sperminNONOate compared to the amount necessary for complete inhibition at 10 kPa. In spite of the high amount of added sperminNONOate, complete inhibition of gill respiration did not occur, and, although respiration rates were reduced, the values were not statistically different from controls (Table 3A, B). The complete inhibitions of gill respiration by NO at 2 kPa, 6 kPa and 10 kPa could be partly neutralized by oxyhemoglobin (Table 3A, see minimum needed oxyhemoglobin concentrations for a maximal neutralization at each PO_2 in Table 3B). Gill respiration after neutralization of the NO inhibition was 42 % lower at 16 kPa, 18 % lower at 10 kPa and 44 % lower at 6 kPa compared to the respective control values (Table 3A). Only at a PO_2 of 2 kPa, gill respiration rates were higher after neutralization with oxyhemoglobin compared to the controls (Table 3A). Added nitrite had no inhibitory effects on gill respiration rates, which were equally high as in the control measurement (Table 3C).

Table 3: (A) Gill respiration rates of Icelandic *Arctica islandica* in nmol O_2 min^{-1} ml^{-1} mg^{-1} WM under control conditions without sperminNONONate, after inhibition by nitric oxide (NO) produced from a defined amount of sperminNONOate, and after neutralization of NO by a defined amount of oxyhemoglobin, mean ± SD. (B) Minimally required concentrations of sperminNONOate and calculated concentrations of released NO in the respiration chamber that completely inhibit gill respiration of *A. islandica* at four different PO_2s, as well as the minimal oxyhemoglobin concentration necessary for neutralization of the inhibition of gill respiration by NO, mean ± SD. At 16 kPa a complete inhibition of gill respiration was not possible, thus no oxyhemoglobin was added (-). (C) Gill respiration rates of Icelandic *A. islandica* under control conditions and after adding 575 µM nitrite to the respiration medium.

A	2 kPa	6 kPa	10 kPa	16 kPa
Control	0.006 ± 0.002	0.025 ± 0.012	0.036 ± 0.028	0.039 ± 0.023
	(n = 3)	*(n = 5)*	*(n = 5)*	*(n = 5)*
SperminNONOate	0.000	0.000	0.000	0.012 ± 0.003
	(n = 3)	*(n = 4)*	*(n = 3)*	*(n = 2)*
Oxyhemoglobin	0.0251	0.014 ± 0.004	0.032	-
	(n =1)	*(n = 4)*	*(n = 1)*	

B	2 kPa	6 kPa	10 kPa	16 kPa
SperminNONOate (µM)	142.7 ± 59.3	467 ± 367.6	1181.3 ± 553.8	> 5900
	(n = 3)	*(n = 4)*	*(n = 3)*	*(n = 2)*
Calculated NO (nM)[1]	22 ± 9	61 ± 60	184 ± 87	> 920
Oxyhemoglobin (µM)	9.5	9.5 ± 1.3	19.2	-
	(n =1)	*(n = 4)*	*(n = 1)*	

[1]calculated NO does not take account of the reaction with O_2, which in aqueous solution is known to react with NO to generate NO_2^-, with the following stoichiometry:
$4\ NO + O_2 + H_2O \rightarrow 4\ NO_2^- + 4\ H^+$. The rate law for this reaction is second order for NO and first order for O_2.

C	6 kPa	10 kPa
Control	0.018 *(n = 1)*	0.022 *(n =1)*
Nitrite	0.017 *(n = 1)*	0.023 *(n = 1)*

Preliminary Discussion

NO production by hemocytes and inhibition of gill respiration

Hemocyte cells of *A. islandica* produced a measurable amount of NO. This NO originated at least partly from NOS activity because its production was inducible by addition of arginine to the sample extract. NOS activity has been detected in the CNS, in peripheral tissues and in hemocyte cells of marine invertebrates such as porifera, cnidaria, crustacea and mollusca (Conte and Ottaviani 1995; Moroz and Gilette 1995; Giovine et al. 2001; Moroz et al. 2004; Palumbo 2005; Jiang et al. 2006).

NOSs from the cnidarian *Discosoma striata*, the mollusk *Aplysia californica* and the hemichordate *Ciona intestinalis* have been cloned and show putative recognition sites for heme, tetrahydrobiopterin, calmodulin, FMN, FAD and NADPH (Palumbo 2005). As we added only arginine to the hemocyte solution to induce NO production, all necessary co-factors for NOS activity must be available at adequate concentrations in hemocyte-isolates of *A. islandica*.

The most applicable method for direct detection of *in situ* NO formation is through electrochemical oxidation of NO on a polarized electrode (Bedioui and Villeneuve 2003; Schreiber et al. 2008), such as the ISO-NOP-electrode. Alternative detection methods have been proposed, such as imaging of NO production using fluorescent dyes such as 4,5-diaminofluorescein (DAF-2) and copper(II) fluorescein (CuFL) (Kojima et al. 1998; Lim and Lippard 2006). Although the CuFL probe detects NO specifically over other reactive species (e.g. H_2O_2, HNO, NO_2^-, NO_3^- and $ONOO^-$) and with a low detection limit of 5 nM (Lim and Lippard 2006), NO production in hemocytes of the ocean quahog was not detectable by CuFL nor DAF-2 at normoxic (21 kPa) or hypoxic (5 kPa, 2 kPa) conditions in our laboratory (data not shown). Possible reasons for the failure to detect NO formation using fluorescent dyes are insufficient specificity of the two dyes, especially of DAF-2, which also reacts with de-hydroascorbic acid and ascorbic acid (Zhang et al. 2002; Lacza et al. 2004). Furthermore, too high background signals may have come from dyes, and finally hemocyte cell concentrations might have been too low to detect NO formation with the dyes. Until now, because of time-limited access to the ISO-NOP-electrode, NO-generation in hemocyte cells could only be measured in one sample, which has to be repeated in future experiments with and without NOS inhibitors (e.g. N^{ω}-Nitro-l-Arginine Methyl Ester = L-NAME) and NO-scavengers (e.g. oxyhemoglobine, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide = PTIO) as well as with different cofactors to ascertain and to characterize NOS activity in *A. islandica*.

Gill respiration of *A. islandica* was completely inhibited at NO concentrations of 22 to 184 nM NO (generated artificially by sperminNONOate) because of a sufficiently high NO stability at low experimental oxygenations of ≤10 kPa. These concentrations are in the range of the NO amounts produced by *A. islandica* hemocytes in the present study, and similar NO concentrations of 30-300 nM NO were recorded in nervous systems of mollusks (Moroz and Gilette 1995). NO lifetime increases at lower PO_2 (Okada et al. 1996). Thus at a PO_2 of 2 kPa an eightfold lower amount of sperminNONOate was enough to completely inhibit gill respiration when comparing to 10 kPa PO_2 conditions. At 16 kPa PO_2, NO was rapidly oxidized and even fivefold higher sperminNONOate addition than at 10 kP could not completely stop oxygen consumption in gills of the ocean quahog. Still, respiration rates decreased to 30 % of the controls. In mammals, mitochondrial respiration is regulated by several effectors such as the availability of ADP to the ATPase, as well as the O_2 and NO equilibrium at cytochrome-*c*-oxidase (Boveris et al. 2000). This appears to equally apply for the ocean quahog. Thus, when internal PO_2 of tissues and hemolymph drops to values ≤ 10 kPa during frequent burrowing periods or > 24 h of shell-closure in *A. islandica* (Taylor 1976; Strahl et al. in revision), accumulating NO may indeed diffuse from hemocytes into tissues and cells and

reduce oxygen binding at complex IV of the mitochondrial respiratory chain and therewith induce a slow-down of metabolic rate in the clams.

Contrary, experimental exposure to even very high nitrite concentrations > 500 µM did not affect gill respiration rates, perhaps because nitrite does not easily enter the cells and mitochondria. Nitrite has been suggested to inhibit aerobic respiration by interacting with cytochrome-*c*-oxidase in denitrifying bacteria (Kucera and Dadák 1983). However, it is also possible that this effect was due to NO generation from nitrite by non-enzymatic acidic reduction (Zweier et al. 1999; Jensen 2007; Schreiber 2009), or by enzymatic reduction via xanthin oxidoreductase (Millar et al. 1998; Gladwin et al. 2005). In order to specify whether or not nitrite is capable to either directly or indirectly inhibit aerobic respiration in *A. islandica*, measurements with mitochondrial isolates or gill tissue with permeabilized membranes and nitrite addition are needed.

Although inhibition of gill respiration in the ocean quahog could be neutralized to some extent by addition of oxyhemoglobin, the reconstituted rates always remained below the initial rates measured prior to the addition of sperminNONOate. So, either a small amount of NO seems to be not available to scavenging by oxyhemoglobin and still acts as metabolic down-regulator, or the gill pieces have suffered some irreversible damage from the experimental treatment. In support of the idea that NO scavenging might not have been completed, Joshi et al. (2002) suggested that oxyhemoglobin rather stores than irreversibly scavenges NO. The authors hypothesized, that oxyhemoglobin may indeed function both as an O_2 and NO carrier and might release NO under hypoxic conditions in mammalian tissues to support vasodilation (Joshi et al. 2002).

Possible sources of nitrate and nitrite in hemolymph and mantle cavity water of A. islandica

In several studies NO production of bivalve hemocytes was indirectly measured by quantifying its oxidation products nitrite (NO_2^-) and nitrate (NO_3^-) (Smith et al. 2000; Novas et al. 2007). More specifically, in aqueous solutions containing oxygen, NO is primarily oxidized to nitrite, and further oxidation to nitrate requires the presence of additional oxidizing agents, such as oxyhemoproteins (Ignaro et al. 1993). On the basis of these findings, we calculated the contents of $\sum$NiNa and also nitrite, only, in hemolymph and mantle cavity water of *A. islandica* that had been incubated 3.5 days at different PO_2, or that had been burrowed for 3.5 days in the sediment.

NO accumulation seemed not enhanced following 3.5 days of hypoxia because nitrite concentrations in the hemolymph of *A. islandica* remained as low as under normoxia. Interestingly, after 3.5 days of anoxic incubation or burrowing (= MRD-clams), the nitrite content was significantly increased in both mantle cavity water and hemolymph. It is unlikely that this indicates increased NO production by NOS, because NOS loses its catalytic activity in the absence of oxygen (Alvarez et al. 2003) and at decreasing pH in tissues and hemolymph (Giraldez et al. 1997). PO_2 in shell water of *A. islandica* was shown to rapidly decline during shell closure and can be assumed to have been

at 0 kPa both, in the hemolymph of anoxically incubated bivalves and in the mantle cavity water of MRD-clams (Strahl et al. in revision). Although the pH of hemolymph and mantle cavity water was not measured in *A. islandica,* tissues might have been slightly acidic after more than three days of anoxia because of anaerobic metabolite accumulation (Strahl et al. 2011; Strahl et al. in revision).

Alternatively, both nitrite and nitrate in hemolymph and mantel cavity water of *A. islandica* may originate from uptake of dissolved inorganic nitrogen (DIN = ammonium + nitrate + nitrite), and only marginally from internal NO production. DIN concentrations of around 45 µM are common in coastal waters of the German Bight (Weigelt-Krenz et al. 2010), and mean ∑NiNa of 34 µM determined in the seawater of the incubation experiment (normoxia) from the inner German Bight near Bremerhaven, match these values. In contrast, the tenfold higher amount of ∑NiNa in the aquarium water of the burrowing experiment may be due to keeping *A. islandica* for several weeks in a closed seawater system. This may favor accumulation of bivalve excretion products to higher values than during the short-term incubation of individual bivalves in the 3-l aquaria for only 3.5 days. Chen et al. (1994) and Cockroft (1990) described the excretion of nitrite and nitrate by the tiger shrimp *Penaeus monodon* and the surf clam *Donax serra* as the intrinsic mechanism for detoxification of ammonia in the blood and for maintaining ionic stability inside the animal.

The conspicuous increase of the nitrite concentration in hemolymph (= 98% of ∑NiNa) and mantle cavity water (= 25% of ∑NiNa) of burrowed and anoxically incubated *A. islandica* potentially resulted from nitrate reduction by facultative anaerobic bacteria, associated with tissues or hemolymph of the clams. Bacteria from the seawater can be taken up by bivalves and accumulate in microbial biofilms attached to the shell, in the gut, or within the soft tissues of these animals (Pujalte et al. 1999; Miller et al. 2006; Rajkowski 2009; Heisterkamp et al. 2010). Facultative anaerobic heterotrophic bacteria (e.g. *Vibrionaceae*) are taken up from the seawater by the oyster *Ostrea edulis* and accumulate in the oysters´ gut and tissues (Pujalte et al. 1999). These bacteria thrive on anaerobic conditions by fermenting carbohydrates and by using nitrate as alternative electron acceptor for their oxidative metabolism (Pujalte et al. 1999). Some microorganisms involved in denitrification or nitrate assimilation can cause a rapid and massive accumulation of nitrite because their nitrite reductase synthesis is repressed or nitrite reduction is inhibited by nitrate (Cole 1990). Such bacteria can be found in the gut of earthworms, where nitrite concentrations are tenfold higher than in the environmental soils (Horn et al. 2003).

Alternatively, *A. islandica* itself may possess nitrate reductase activity in its tissues, which produces nitrite under anoxic conditions. Nitrate reductase activity has already been detected in cells of humans, mice and rat, originating from a xanthine oxidoreductase (XOR), but possibly also from other enzymes (Jansson et al. 2008). Both, prolonged hypoxia and acidosis increase expression levels and the nitrate reducing capacities of XOR, which suggest increased nitrite formation under these conditions (Kelley et al. 2006; Jansson et al. 2008).

In parallel to the increase in nitrite content in the hemolymph of the anoxic *A. islandica*, the nitrate content of the anoxic incubation water decreased by a corresponding amount. This effect was only visible during the incubation experiment with bivalves individually incubated in small seawater volumes of only 3 l. The permanently open siphons of the animals allowed for intensive turnover of mantle cavity water in exchange with the aquarium water. Apparently, nitrate was absorbed from the aquarium water and was reduced to nitrite within the bivalve mantle cavity water. In contrast, during the burrowing experiment, normoxic- and MRD-clams where kept all together in three inter-connected 60-l tanks with re-circulating seawater, which may have masked the small absorption of nitrate.

Nitrite and NO-signaling under anoxia – a hypothesis

While nitrite is usually considered to be an oxidative metabolite derived from NO, it can also be a storage pool for NO formation (Cosby et al. 2003). In mammals and ectotherms NO can be generated from nitrite by non-enzymatic acidic reduction (Samouilov et al. 1998; Zweier et al. 1999; Jensen 2007), or by enzymatic reduction via xanthin oxidoreductase (Millar et al. 1998; Gladwin et al. 2005; Jansson et al. 2008). Both reactions are favored at low pH and low PO_2 (Samouilov et al. 1998; Zweier et al. 1999), as well as at high c[NO_2^-] (Jensen 2007). During prolonged anoxic incubation or during shell closure in the burrowed status, intracellular acidosis in *A. islandica* may result from accumulation of anaerobic metabolites (Oeschger 1990; Strahl et al. 2011; Strahl et al. in revision), probably leading to a highly reduced state that could support the reduction of nitrite to NO, comparable to NO generation during ischemia in mammals (Zweier et al. 1999).

Aerobic ATP production is already null in anoxic tissues so that during prolonged anoxia in *A. islandica* NO may rather function as a cellular protection than as a metabolic regulator. When the bivalves return to the surface after burrowing, tissues are re-oxygenated. But in contrast to several vertebrate and invertebrate species undergoing dormant states (Hermes-Lima and Zenteno-Savín 2002; Ramos-Vasconcelos and Hermes-Lima 2003; Lushchak et al. 2005; Larade and Storey 2009; Zenteno-Savín et al. 2010), *A. islandica* exhibit neither a ROS-burst after hypoxia-reoxygenation, nor up-regulates antioxidant capacities during prolonged MRD (Strahl et al. 2011; Strahl et al. in revision). This is possibly due to an alternative oxidase in *A. islandica* mitochondria which has a higher P_{50} than cytochrome-*c*-oxidase and provides a phosphorylation-independent bypass for electrons (Tschischka et al. 2000; Abele et al. 2007). The alternative oxidase is less sensitive to NO than the cytochrome-*c*-oxidase (Millar and Day 1996), which may still be inhibited by NO at beginning reoxygenation. In lowering tissue PO_2 and minimizing reduction of respiratory chain intermediates, the alternative oxidase may reduce the risk of ROS formation on reoxygenation in *A. islandica* tissues (Tschischka et al. 2000). Probably this reaction is more pronounced at high cellular NO concentrations.

In conclusion NO may have several functions in physiological processes of the ocean quahog such as induction of metabolic down-regulation at internal PO_2 levels below 10 kPa, as well as for cellular defense and oxidative burst avoidance during surfacing and reoxygenation after prolonged hypoxia and anoxia. NOS-like activity was detected in hemocyte cells of the clam, but further physiological and genetic studies are needed to confirm and characterize NOS-activity and to identify possible non-enzymatic NO formation processes in hemocytes and tissues of *A. islandica*.

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5.2. Hemocyte cell cultures

Bivalves have an open blood system and the hemolymph, mainly consisting of plasma and hemocyte cells, circulates within the vascular system and in the connective tissues of the molluskan inner organs (Morse and Zardus 1997). Molluskan hemocyte cells serve a variety of important functions such as wound and shell repair, nutrient digestion and transport, excretion and internal defense (Cheng 1984). Appearance and 'behavior' of hemocyte cells are highly variable (Cheng 1984). They can be found freely floating in the hemolymph or attached to tissue surfaces. They can be roundish or flattened and spread out when developing filopodia (also called pseudopodia) to perform amoeboid cell movements. They may occur as single cells, may be interconnected loosely to other hemocytes or appear in dense aggregations of several hundred cells (Fig. 1A, B, C). A presently open question is the origin of hemocyte cells.

Although it is supposed that hemocyte cells arise from differentiating connective tissue cells (Cheng 1984), no stem cells have been described so far and no cell line of differentiation has been defined to prove this assumption. Approaches to culture molluskan cells, including hemocytes, have had limited success (Mialhe et al. 1995; Power et al. 1998; Cao et al. 2003). Possible reasons herefore are the low speed of hemocyte cell proliferation and the lack of knowledge about specific growth factors (Lebel et al. 1996; Canesi et al. 2000; Cao et al. 2003). The alternative to culturing the cells is to keep hemocytes cells alive and vital as long as possible in primary cultures under controlled conditions with respect to temperature, pH, osmolarity and nutrient content.

The present study aims to optimize primary cell culture conditions of hemocyte cells using the ocean quahog *Arctica islandica*. Herefore, hemolymph was drawn from the adductor muscles of live *A. islandica* from the German Bight, using sterile needles and 10-ml-syringes. The hemolymph was centrifuged for 10 min at 450 x g at room temperature (20°C) and the pellet containing the hemocytes was resuspended in different solution mixtures (Table 1, ID 1, 2, 9). In alternative approaches, hemolymph and hemocytes were left in their natural state (Table 1, ID 3-8). Resuspended hemocytes or natural hemocytes in the hemolymph were cultured for a minimum of 20 days under 9 different cell culture protocols at two different temperatures (Table 1). Between 12,500 and 1 mio hemocyte cells were cultured in a total medium volume of 0.5 ml to 10 ml (Table 1). Hemocyte cells were either cultured in coated (Table 1, ID 3 and 5-9) or non-coated culture flasks or wells (Table 1, ID 1, 2, 4), and the culture media was exchanged every three days for two cultures only (Table 1, ID 1, 2). In all other cultures the hemocytes cells stayed in the same culture media for the entire experimental time (Table 1, ID 3-9). The optimal conditions for *A. islandica* hemocytes maintenance and viability were determined by microscopic observation of each cell culture and were checked every other day for the entire experimental period. Additionally pictures were taken every other day to estimate percentages of healthy and dying hemocytes. Criteria for healthy hemocyte cells included the development of filopodia, attachment to the plastic of the culture flask, and a cell diameter of less than 50 µm in single free floating cells. In contrast, dying cells were identified as perfectly round morphs without pseudopodia and diameters exceed-

ing 50 µm, and were typically floating freely in the media before cell lysis. Percentage of healthy and dying cells was calculated from pictures, which were taken.

Table 1: Cell culture conditions of hemocytes of *Arctica islandica* from the German Bight. Temp = cell culture temperature, $Vol_{Leibovitz}$ = volume of Leibovitz-medium in the culture medium in °C, Vol_{Alseve} = volume of Alseve-buffer in the culture medium in ml, Vol_{Hemol} = volume of hemolymph in the culture medium in ml, Vol_{Medium} = total volume of the culture medium in ml, $Cells/Vol_{Medium}$ = cell counts in the total volume of the culture medium in ml, Centrifugation = Hemolymph-sample was centrifuged 10 min at 450 x g and room temperature and hemocytes resuspended in Leibovitz-medium (ID 1 and 2) or a mixture of Leibovitz-medium and Alseve-buffer (ID 9), Coated = cell culture flasks (ID 1-4) or wells (with a volume of 0.5 ml-2 ml, ID 5-9) were coated with poly-D-lysine, Changed = Leibovitz-medium was exchanged every 3 days. 'X' = hemolymph-sample was centrifuged, culture flasks or wells were coated or medium was exchanged. '-' = hemolymph-sample was not centrifuged, culture flasks or wells were non-coated, medium was not changed, solutions were not in the cell culture medium.

ID	Temp	$Vol_{Leibovitz}$	Vol_{Alseve}	Vol_{Hemol}	Vol_{Medium}	$Cells/Vol_{Medium}$	Centrifugation	Coated	Changed
1	18	10.00	-	-	10.00	1 mio	X	-	X
2	6	10.00	-	-	10.00	1 mio	X	-	X
3	6	5.50	1.00	3.50	10.00	1 mio	-	X	-
4	6	5.50	1.00	3.50	10.00	1 mio	-	-	-
5	6	0.25	0.18	0.07	0.50	12,500	-	X	-
6	6	0.50	0.36	0.14	1.00	25,000	-	X	-
7	6	0.75	0.54	0.21	1.50	37,500	-	X	-
8	6	1.00	0.72	0.27	1.99	50,000	-	X	-
9	6	1.60	0.40	-	2.00	1 mio	X	X	-

Best cell viability was observed when directly transferring 3.5 ml of fresh hemolymph containing around 500,000 hemocyte cells to a sterile cell culture flask (≈140,000 cells ml^{-1}), either non-coated or coated with poly-D-lysine and containing a solution mixture of 5.5 ml Leibovitz-medium (Leibovitz L-15, filtered sterile and modified by 20.2 g l^{-1} NaCl, 0.54 g l^{-1} KCl, 0.6 g l^{-1} $CaCl_2$, 1 g l^{-1} $MgSO_4$, 3.9 g l^{-1} $MgCl_2$, 20.8 g l^{-1} glucose, 100 units ml^{-1} penicillin G, 100 µg ml^{-1} streptomycin, 40 µg ml^{-1} gentamycin, 0.1 µg ml^{-1} amphotericin B, 10 % foetal calf serum, pH 7, 1000 mOsm, Novas et al. 2007) and 1.5 ml Alseve-buffer (20.8 g l^{-1} glucose, 8 g l^{-1} sodium citrate, 3.36 g l^{-1} EDTA, 22.5 g l^{-1} NaCl, pH 7, 1000 mOsm, Novas et al. 2007) at 6 °C room temperature (see Table 1, ID 3 and 4). After one day at these conditions more than 90 % of the hemocyte cells were attached to the coated or uncoated plastic of the culture flasks. Hemocytes spread out and flattened, formed filopodia and show amoeboid cell movements. Less than 10 % of the cells were floating freely or forming aggregations. After 17 days, more than 90 % of hemocyte cells were still attached. Between day 19 and day 23 of cell culturing, the number of free floating hemocyte cells

with diameters exceeding 50 µm and underwent cell lysis, increased to more than 30 %. In the other media (Table 1, ID 1, 2 and 4-9), a higher percentage of hemocyte cells were free-floating with diameters exceeding 50 µm after a few days (e.g. ID 1: more than 50 % of hemocytes after 4 days, ID 2 and 9: more than 30 % of hemocytes after 9 days) and showed higher percentages of cells in lysis throughout the entire maintenance period (e.g. ID 1: more than 80 % dead cells after 7 days).

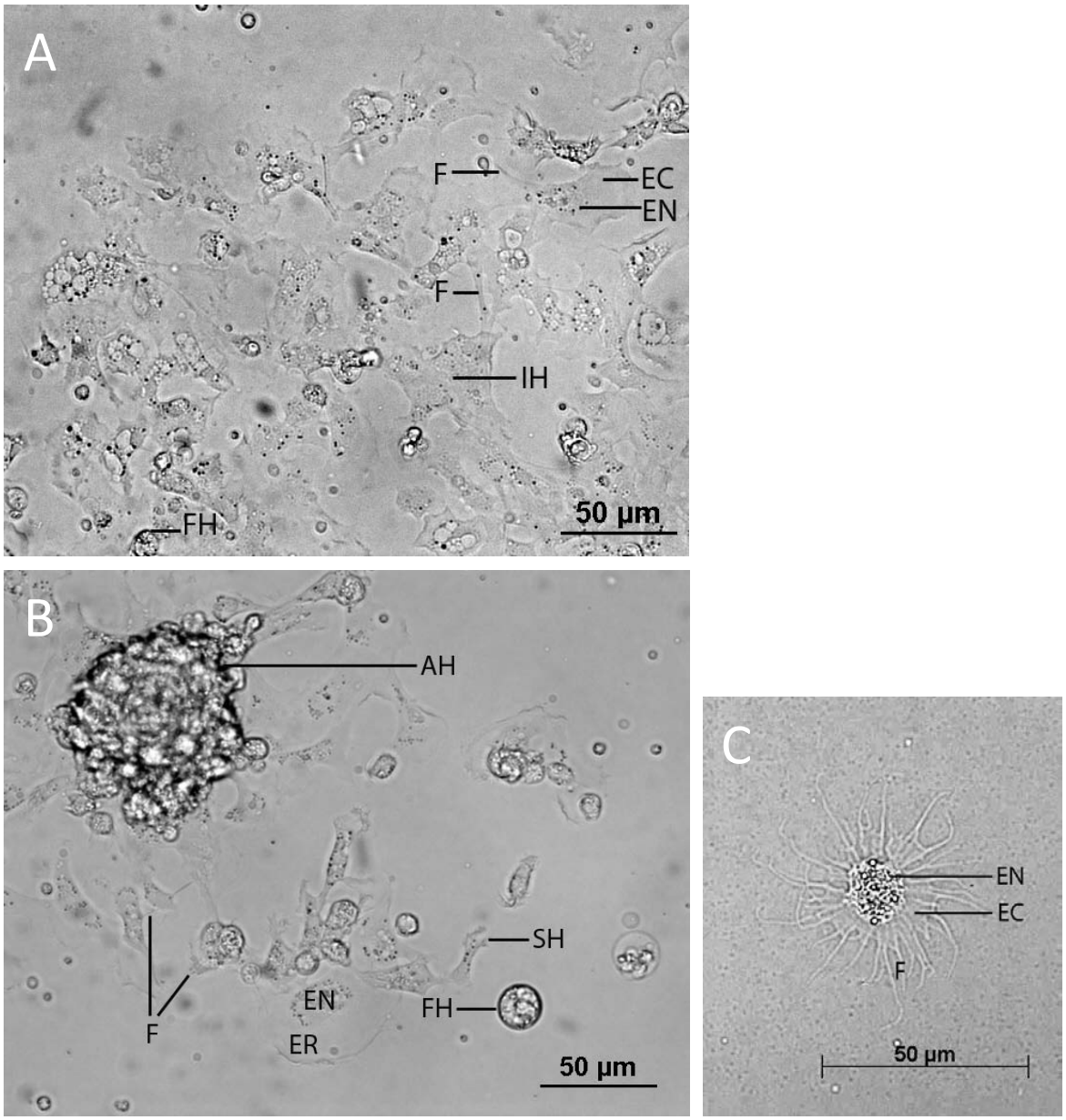

Fig. 1: Hemocyte cells of *Arctica islandica* from the German Bight. (A) flattened hemocyte cells, fully attached to the cell culture flask after 3 days, (B) after 6 days aggregated and (C) Single hemocyte cell with pseudopodia, attached to the plastic of the culture flask, after 1 day in a cell culture (solution mixture of hemolymph, Leibovitz-medium and Alseve-buffer at 6°C; see text). AH = aggregated hemocytes, EC = ectoplasm, EN = endoplasm with cell organelle, F = filopodia, FH = free floating hemocyte with a perfectly round cell shape, IH = interconnected hemocytes, SH = single hemocyte cell.

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6. DISCUSSION

In the following section the data presented in chapter III (publication I-V) and in the section 'additional results' (5.1) will be discussed.

6.1. What contributes to the long life expectancy in *Arctica islandica*?

One of the most widely accepted and over the past 50 years supported theory of aging is the 'Free radical theory of aging' by Harman (1956), which implies a negative correlation between rates of oxidative stress, age-related disease and longevity in animals (reviewed in Salmon et al. 2010). In recent years the straight forward relationship between ROS production rates, antioxidant capacities and lifespan is under discussion, although there is some support for the connectivity between life-history and the metabolic rate in vertebrate and invertebrate species (Buttemer et al. 2010; Constantini 2010; Salmon et al. 2010).

The presently identified low species-specific metabolic rates, low ROS formation rates and the well-established antioxidant defense system in *A. islandica* are strong arguments for extreme longevity (Abele et al. 2008; publication I, III, IV) and support the aging theories by Pearl (1928) and Harmann (1956). However, a MLSP of the ocean quahog which is 10-100 times higher than that of most other bivalve species (Powell and Cummins 1985; Philipp 2005; Estabrooks 2007) cannot be explained solely by relatively low routine rates of metabolic ROS production. One of the reasons herefore is that ROS formation rates of mitochondrial isolates from *A. islandica* range among - but not below - the lowest values observed in other bivalves under normoxic conditions (Buttemer et al. 2010). In addition, environmental conditions as well as species-specific behavior and physiological parameters in the cell seem to play a major role in the reduction of oxidative stress in *A. islandica* and may be crucial for retarded cellular aging and extend lifetime in the ocean quahog. In fact, Constantini (2010) found that cellular levels of oxidative stress depend on formation rates of radicals, as well as on structural and chemical defense mechanisms in the cell. Chemical defense mechanisms include antioxidants, molecular repair (e.g. DNA-repair), elimination of damaged cells (apoptosis) and cell renewal (proliferation).

Under normoxic resting conditions values of the mean standard metabolic rate (SMR) in *A. islandica* are already below the average measured values of 58 different bivalve species (publication IV). Moreover, H_2O_2 formation in the coupled state 3 and uncoupled state 4 in isolated mitochondria is three to five times lower in *A. islandica* than in the shorter-lived mud-burrowing clams *Mya arenaria* (MLSP = 18 years, pers. comm. A. Sukhotin) and *Laternula elliptica* (MLSP = 36 years, Philipp et al. 2006) (Buttemer et al. 2010). Only the ROS formation in mitochondrial isolates from the epibenthic and short-lived scallop *Aequipecten opercularis* (MLSP = 8-10 years, Heilmayer et al. 2004) is about 10 times lower under state 4 conditions when compared to *A. islandica* (Buttemer et al. 2010), although standard metabolic rates are definitely higher in the scallop. Thus, *A. islandica* and *A. opercularis* represent models of two completely different life strategies. *Aequipecten opercularis*

performs burst swimming activity, during which ROS formation and oxidative damage, particularly in the mitochondria, are anticipated to be higher than at SMR. To conserve mitochondrial intactness and to stabilize routine ROS production over lifetime, damaged mitochondria are permanently eliminated from the cells in *A. opercularis* (Philipp et al. 2008). Moreover, damaged cells are eliminated and replaced through high programmed cell death (= apoptosis) and proliferation (publi-cation I).

In contrast, *A. islandica* is rather inactive and is suggested to reduce lifetime ROS formation by lowering metabolic rates. *Arctica islandica* regulates mean internal PO_2 to protective values of < 5 kPa under normoxic environmental conditions and to 0 kPa during prolonged shell closure and MRD for longer than 24 h (Abele et al. 2010; publication III). Typically, the clams live burrowed in the sediment and frequently enter a metabolically depressed state, which can last for several days (Taylor 1976; publication III). As an oxyconform species, *A. islandica* is suggested to decrease respiration rates as PO_2 declines (Dejours 1981), which might also lower cellular ROS-formation. In agreement, H_2O_2 formation in isolated gill tissue was found to be 10 times lower at 5 kPa than at 21 kPa (publication III) and supposedly fully subsided at 0 kPa. Accordingly, a lifetime H_2O_2 formation in *A. islandica* can be expected to be much lower than rates that would be calculated based on the measurements with mitochondrial isolates at 21 kPa (normoxia). In fact, the accumulation of protein oxidation products over age and lipofuscin accumulation in *A. islandica* tissues were extremely low when compared to clams and scallops of shorter MLSPs. Cell turnover rates remained constant until 150 years of age, but were much lower than in tissues of *A. opercularis* (Strahl et al. 2007; Philipp and Abele 2010; publication I).

The generally low mitochondrial ROS formation in *A. islandica* is complemented by well-established cellular protective mechanisms (i.e. SOD- and CAT-activities, GSx content) which are typically maintained at constantly high levels for over 200 years of lifetime (Abele et al. 2008). Stable expression rates of antioxidants seem crucial and perhaps even more essential to reach extremely old age than the absolute level of the antioxidants. Tissue specific antioxidant capacities were found to range among - but not above - the highest values observed in clams, mussels and scallops of shorter lifespans (Ballantyne and Berges 1991; Viarengo et al. 1991; Gamble et al. 1995; Viarengo et al. 1995; Sukhotin et al. 2002; Philipp et al. 2005b; Philipp et al. 2005a; Abele et al. 2008). But in contrast to *A. islandica*, in many organims, including humans, rats, insects and also in marine bivalves, antioxidant capacities are high in younger individuals but decline dramatically over lifetime (Sohal et al. 1987; Sanz et al. 1996; Canesi and Viarengo 1997; Hernanz et al. 2000; Philipp et al. 2005a; Philipp et al. 2005b). In the Antarctic clam *L. elliptica* and in the scallops *Adamussium colbeckii* and *Aequipecten opercularis*, either the GSx content or the SOD and CAT activity decreased over the total lifetime. The decline was even more pronounced in the shorter-lived temperate scallops than in the longer-lived polar species (Philipp et al. 2005a; Philipp et al. 2005b).

While cellular protein- and lipid-oxidation rates can be reduced by effective antioxidant protection, the damage of nuclear and mitochondrial DNA has to be quickly repaired in order to prevent rapid loss of functioning in cells, tissues and the entire organism. Data on damage and repair rates of nuclear and mitochondrial DNA in *A. islandica* are not available. However, DNA damage intensity is known to vary considerably between bivalve species, while DNA repair capacity generally seems to decline with age (Accomando et al. 1999; Dixon et al. 2000; Pruski and Dixon 2003). Higher DNA damage and lower DNA polymerase activity (= DNA repair activity) was found in older age classes of *M. edulis* (Accomando et al. 1999) and in old hydrothermal vent bivalves *Bathymodiolus azoricus* (Pruski and Dixon 2003) when compared to younger individuals. DNA-damage (e.g. oxidation of bases, strand breaks or lesions) can lead to transcription errors, false and mutated transcription products and ultimately to reduced cell viability (Philipp and Abele 2010). If not repaired, a large amount of DNA damage can accumulate and induce apoptosis (Pruski and Dixon 2003). In *A. islandica,* both, proliferation rates and apoptosis intensities were lower in mantle, gill and adductor muscle tissue than in tissues of the short-lived *A. opercularis*, and remained at constant levels for ages between 7 and 148 years (publication I). These slow but constant cell turnover rates complement the low basal ROS formation and probably also low DNA-damage, the intermittent reduction of metabolic rates and the constant antioxidant protection over lifetime, and are in support of the negligible senescence in *A. islandica*.

The only symptoms of physiological change over age observed in the ocean quahog were decreasing cell turnover rates in the heart muscle over a lifetime of 140 years (publication III). Presently it is not clear, whether this indicates higher damage levels and possibly arising loss of functioning in the heart of aging *A. islandica* due to decreasing cell renewal capacities. Impaired cardiac function has been suggested to limit lifespan in long-lived mammals (Leri et al. 2003) and perhaps 'heart failure' could finally cause natural death in very old ocean quahog. However, decreasing cell turnover rates could also indicate lower rates of waste product accumulation (e.g. lipofuscin, protein-carbonyles, DNA-damage) and cell damage in the heart tissue of *A. islandica* over lifetime and thus, a reduced need for cell renewal.

In conclusion, a 'calm' lifestyle and high cellular maintenance favor a MLSP of several hundred years in the ocean quahog. *Arctica islandica* is characterized by slow growth rates, late sexual maturity, an inactive lifestyle with generally low metabolic activity, sustained protection from oxidative injury over age along with low accumulation of oxidative damage products and slow but continued cell turnover rates (Thorarinsdóttir and Steingrimsson 2000; Strahl et al. 2007; Abele et al. 2008; publication I, IV) (Table 6.1). All of these traits in *A. islandica* require constant, but far less energy investment over time than the short-lived bivalve *A. opercularis*. A very prominent and species-specific energy-saving mechanism in *A. islandica* is its behavior of self-induced MRD which is supposed to be one of the key life-prolonging parameters in the ocean quahog (publication III). Furthermore, like other long-lived mollusks, the ocean quahog seems to budget lifetime energy investments for somatic growth and maintenance and persistent but slow rates of gametogenesis

(Arntz et al. 1994). Rowell et al. (1990) and Thórarinsdóttir (2000) investigated the annual gametogenic cycle of *A. islandica* in coastal populations of South West Canada and North West Iceland. Both studies found that spawning in the clams occurred throughout the entire year.

Table 6.1: Life history traits in the long-lived mud clam *Arctica islandica* and the short-lived scallop *Aequipecten opercularis*, MRD = metabolic rate depression. Data sources: [1]Wanamaker et al. 2008, [2]Heilmayer et al. 2003, [3]Jenkins et al. 2003, [4]Heilmayer et al. 2004, [5]Thorarinsdóttir and Steingrímsson 2000, [6]Román et al. 1996, [7]publication I, [8]Philipp et al. 2006, [9]Abele et al. 2010, [10]Abele et al. 2008, [11]Buttemer et al. 2010, [12]Strahl et al. 2007, [13]Brey et al. 1990, [14]Schmidt et al. 2008.

Arctica islandica	*Aequipecten opercularis*
long-lived: > 400 years[1]	short-lived: 10 years[2]
modest energy throughput over lifetime	high energy throughput over lifetime
inactive lifestyle: mud-burrowing	active: swimming[3]
slow growth[12]	fast growth[4]
late sexual maturation: at ages between 10-30 years[5]	early sexual maturation: at ages < 1 year[6]
modest investment into reproduction over long time spans[5]	high investment at peak-reproductive events[6]
low standard metabolic rates, declining during frequent MRD[7]	high standard metabolic rates, increasing when swimming[8]
low mean internal PO_2: < 4 kPa, 0 kPa during MRD for > 24 h[9]	higher mean internal PO_2: > 6 kPa, never 0 kPa[9]
high antioxidant protection, constant over 200 years of age[10]	high antioxidant protection, decreasing over age[8]
low basal ROS formation, lower during MRD[11]	low ROS formation[8]
low accumulation rates of oxidative damage over age[12]	high accumulation rates of oxidative damage over age[8]
low cell turnover-rates, constant over age[7]	high cell turnover-rates, no data available for information over age[7]
low predation pressure at old ages[13]	high predation pressure also at old ages[14]

Although spawning of *A. islandica* was most intense from June to August in Iceland and from July to September in Canada, nearly all stages of gametogenesis (such as early active, late active, ripe, partially spawned and spent) could be found at all times of the year (Rowell et al. 1990; Thorarinsdóttir 2000). This might prevent the occurrence of a seasonal peak of energy investment into reproduction and lead to a shift in energy allocation between somatic and reproductive tissues in *A. islandica*, which might be connected with increasing rates of oxidative damage in tissues of bivalves (C. Guerra, pers. comm.). Temporarily high energy investments into reproduction are supposed to induce increasing rates of protein and lipid-oxidation in tissues of the extremely short-lived scallop *Argopecten ventricosus* (C. Guerra, pers. comm.) (MLSP = 2-3 years, Peña 2001). In this

species spawning occurs twice per year and gametogenesis represents a mayor energetic drainage, which is fueled by energy storages in tissues, mainly in the adductor muscle. Similarly, in *A. opercularis* the energy requirements related to gametogenesis involve the consumption of energy resources resulting in the reduction of the condition indices (Román et al. 1996). Opposite to the long-lived *A. islandica*, *A. opercularis* is a model species representing a life strategy with high energy investments over lifetime and early 'signs of wear and tear' (Table 6.1). The short-lived scallop is characterized by fast growth, an active life-style, high standard metabolic rates, early sexual maturation, high energy investment into peak reproductive events, decreasing cellular defense mechanisms, increasing antioxidant damage over lifetime and high cell turnover rates (Román et al. 1996; Jenkins et al. 2003; Heilmayer et al. 2004; Philipp et al. 2006; publication I) (Table 6.1).

6.2. Metabolic rate depression in *Arctica islandica* and the possible role of NO in regulating cellular respiration in this species

Arctica islandica perform a species-specific behavior of frequent and, yet, unpredictable burrowing into deeper sediment horizons and of self-induced MRD, which seems to be a key life prolonging strategy in the ocean quahog. In several vertebrate and invertebrate species metabolic down-regulation has mostly been linked to unfavorable environmental conditions, suboptimal food availability or escape from predators (Stuart and Brown 2006; Bickler and Buck 2007; Zenteno-Savín et al. in press). In the present study, *A. islandica* was not only burrowed during winter in Iceland field experiments (= 100 % of all investigated animals), but to a lower percentage (= 37 % of all investigated animals) also in summer during the main feeding season (publication III). In laboratory aquarium tanks, when deprived of their sedimentary retreat, some clams spontaneously closed the shells for periods longer than 24 h and induced MRD at constant temperatures and food *at libitum* representing stable environemental conditions (publication III). Therefore, a dormant state in *A. islandica* might be triggered to a greater extent by internal than by environmental cues. The inductive mechanism of MRD in *A. islandica* is unexplored, but the signaling molecule NO could play an important role in this process. In the present work, isolated hemocyte cells were found to produce a measurable amount of NO under low oxygen conditions (additional results, 5.1). This NO is able to diffuse into cells and tissues and to inhibit cytochome-*c*-oxidase and, if concentrated in high enough amounts, to reduce or fully inhibit mitochondrial respiration. Indeed, in *A. islandica* the respiration of isolated gill tissues was completely inhibited at low experimental $PO_2 \leq 10$ kPa by NO concentrations in the range of NO amounts produced by hemocytes (additional results, 5.1).

In the metabolically depressed state, ROS formation is considerably reduced when compared to normoxia, and supposedly decreases to zero as respiration stops (publication III). However, not only the overall time that animals spend in MRD is important for their lifetime ROS balance and the intensity of oxidative stress. Also the mitochondria need to show certain adaptations to pronounced fluctuations of the shell water and tissue PO_2 and especially to tissue reoxygenation after MRD.

Thus, a ROS-burst was absent in isolated gill-tissues of *A. islandica* after hypoxia (5 kPa)-reoxygenation (21 kPa)-experiments and, consequently, no induction of antioxidant capacities (i.e. SOD- and CAT-activity, GSx-content) was observed during 3.5 days of either self-induced or forced MRD (publication II, publication III). This contrasts several findings in vertebrates and invertebrates undergoing dormant states. Higher levels of free radical damage during awakening and tissue reoxygenation are reported for hypoxia- and anoxia-tolerant invertebrates that undergo estivation, hibernation or freezing (Hermes-Lima and Storey 1995; Ramos-Vasconcelos and Hermes-Lima 2003). This is probably due to increased energy demands and respiration rates for disposal of anaerobic metabolites and recharging of the phosphagen and ATP pools (Herreid 1980; Ellington 1983). In most of these species, MRD appears to trigger antioxidant functions to reduce hypoxia-reoxygenation injury during emergence from MRD (Boutilier and St-Pierre 2000; Hermes-Lima and Zenteno-Savín 2002; Lushchak et al. 2005; Larade and Storey 2009). Activities of ROS-detoxifying enzymes SOD and CAT and content of selenium-dependent glutathione peroxidase increased 1.6 to 2.2-times in tissues of estivating land snails *O. lactea* and up to 4.9-times in *H. aspera* after 20 days of dormancy when compared to active snails (Hermes-Lima and Storey 1995; Ramos-Vasconcelos and Hermes-Lima 2003). Similar adaptations can be found in diving birds and mammals when compared to non-diving species, where enhanced basal antioxidant levels prevent oxidative injury by post-dive ROS production during ischemia/reperfusion (Vàzquez-Medina et al. 2007; Vázquez-Medina et al. 2010; Zenteno-Savín et al. 2010).

In *A. islandica*, the avoidance of ROS seems to be a key mechanism to deal with the potential problem of strongly fluctuating internal PO_2 associated with burrowing and shell closure. Anaerobic metabolism in *A. islandica* sets on at very low PO_2 <2 kPa and MRD helps to avoid an early accumulation of acidic anaerobic metabolites in cells and tissues. Furthermore, even at lower PO_2 than 2 kPa, the onset of anaerobiosis can be protracted if the metabolic requirements are small enough. Thus, an anaerobic energy production was not observed in the following 24 h at 0 kPa in the shell cavity water of *A. islandica*. Finally, succinate content in the adductor muscle of the clams rose after 3.5 days of anoxic incubation or burrowing (publication II, publication III). Reduced production of anaerobic end-products in *A. islandica* limits the need for enhanced recovery respiration during surfacing and minimizes the risk for enhanced ROS formation. Other physiological parameters possibly involved in lowering ROS formation rates during reoxygenation are uncoupling proteins (UCPs) which promote the opening of the proton leak (= non-phosphorylating reflux of protons from the mitochondrial intermembrane space into the matrix), or the alternative oxidase (Stuart and Brown 2006; Abele et al. 2007). Proton leak prevents extensive ROS formation by lowering the proton motive force and the membrane potential (Brand 2000, Philipp 2005), whereas alternative oxidases increase the rate of cellular oxygen consumption and thereby lower tissue PO_2 (Tschischka et al. 2000; Abele et al. 2008). Respiration measurements of isolated mitochondria support the existence of an alternative oxidase pathway in cells of *A. islandic*a, which provides a posphorylation-independent by-pass for electrons (Tschischka et al. 2000). The alternative oxidase has a higher P_{50} than cytochrome-*c*-oxidase and further, the activity of this alternative pathway may

be favored by NO, which is possibly produced in tissues of *A. islandica* during prolonged MRD and anerobiosis (additional results, 5.1). The alternative oxidase is less sensitive to NO than the cytochrome-*c*-oxidase (Millar and Day 1996), which might be still inhibited by NO at the beginning reoxygenation process after MRD.

6.3. What are the reasons for the differences in MLSPs in geographically separated *A. islandica* populations? Intrinsic vs. extrinsic determinants.

Arctica islandica is one of the few known species with conspicuous discrepancies in MLSP of geographically separated populations: Iceland MLSP = 407 years (Wanamaker et al. 2008), Helgoland MLSP = 150 years (Witbaard and Klein 1994; Witbaard et al. 1999; Epplé et al. 2006), Norway MLSP = 93 years (publication IV), Kattegat MLSP = 71 years (publication V), White Sea MLSP = 53 years (publication V), Kiel Bay MLSP = 40 years (Brey et al. 1990; Begum et al. 2010).

Reported maximum life spans in wild populations must always be viewed with caution because their determination depends largely on the chance of sampling the 'oldest' individual in a population (Beukema et al. 2002; Philipp and Abele 2010). However, clear differences in MLSP exist between geographically separated *A. islandica* populations and the collected amounts of animals used for age determinations are sufficiently high for reliable estimations of MLSP's, at least for the populations from Iceland, German Bight and Kiel Bay (several hundred for each population, see also Brey et al. 1990; Witbaard and Klein 1994; Steingrimsson and Thorarinsdóttir 1995; Witbaard 1997; Witbaard et al. 1999; Thorarinsdóttir and Steingrimsson 2000; Zettler et al. 2001; Schöne 2003; Schöne et al. 2003; Schöne et al. 2004; Schöne et al. 2005a; Schöne et al. 2005b; Thorarinsdóttir and Jacobson 2005; Epplé et al. 2006; Strahl et al. 2007; Abele et al. 2008; publication I, IV, V).

Throughout the literature, there is substantial agreement that the heritage of life expectancy in an individual is of low probability (around 33 % in humans), whereas the remaining variability can be mainly attributed to the environment (Barja 2008). Thus the genetic basis of a maximum life expectancy may be the same for all *A. islandica* populations, and the findings of highest maximum ages at Iceland may be on account of better living conditions for the clams when compared to other regions. In agreement Held and co-workers detected low genetic differentiation between populations from the Kattegat, Helgoland, the Baltic Sea and Norway and also between Iceland and White Sea populations (Held, pers. comm.).

Seawater temperature, salinity, food availability and anthropogenic impacts, such as pollution or commercial fisheries, seem to be the main environmental parameters affecting MLSPs in *A. islandica* (see also Table 6.2) with elevated environmental stress levels being correlated with lower life expectancies in *A. islandica* populations (publication I, V).

Lowest MLSPs of mud clams were found in the White Sea and the Kiel Bay (= 10 times lower than Icelandic *A. islandica*), which are regions with very low salinities and relatively strong hydrographic fluctuations (Table 6.2). High fluctuations in salinity may cause severe changes in respiration rates as well as in protein and RNA synthesis and force the bivalves to invest more energy into iso-osmotic regulation and cell-volume regulation by ATP-dependent pumps, such as the Na/K or the Na/Cl pump (Berger and Kharazova 1997). Thus, presumably the energy-requirements from the elevated saline stress may be responsible for the shortened the life spans in the fully marine *A. islandica* from Kiel Bay and White Sea. Similarly, during long-term laboratory experiments, the mortality of Kiel Bay *A. islandica* increased with lower salinities, whereas growth rates decreased (E. Philipp, pers. comm.).

MLSP was 3 to 4 times lower in the warm-adapted German Bight populations than in the Iceland populations, probably because of higher annual temperature fluctuations in the North Sea (4-19°C) and strong anthropogenic impacts such as fishery activities causing high amounts of shell damage (Witbaard and Klein 1994; Rumohr and Kujawski 2000), frequent events of eutrophication in the last 60 years (Rijinsdorp and van Leeuwen 1996) and steadily increasing water temperatures over the past 40 years (Wiltshire and Manly 2004) (Table 6.2). Similar environmental conditions may also cut on the lifespan of Kattegat mud clams (Pearson et al. 1985; Pihl 1994) (Table 6.2). In marine ectotherms, the metabolism is directly affected by sea water temperatures (Clarke 2003), causing higher metabolic rates and probably higher ROS formation rates over lifetime at increasing temperatures (Abele et al. 2002). Indeed, mass-specific metabolic activity of *A. islandica* was significantly higher at increased experimental temperatures, at least in four of six investigated populations (publication IV, V). In contrast, narrow, cold thermal windows along with limited food supply, for example during winter periods (Gudmundsson 1998), are suggested to reduce metabolic rates and presumably induce higher frequencies of self-induced MRD in Iceland mud clams (publication III). This, together with less anthropogenic impacts around Iceland (Witbaard et al. 1999; Kaiser et al. 2006), provides significantly lower cellular damage over lifetime and supports extreme longevity in the Iceland population of *A. islandica* (Table 6.2).

Antioxidant protection levels over lifetime were comparable in the six investigated populations (publication V), and proliferation rates and apoptosis intensities showed similar values for German Bight and Iceland clams. However, accumulation rates of the age pigment lipofuscin, a marker for oxidative cell damage (Brunk and Terman 2002a; Brunk and Terman 2002b; Philipp et al. 2005b; Philipp et al. 2006), suggest stress levels of different environments of the ocean quahog to be negatively correlated with MLSP of different *A. islandica* populations of the NE Atlantic (Abele, Basova, Philipp, Strahl, Sukhotin, unpublished data). Highest lipofuscin accumulation rates were found in mantle, gill and adductor muscle tissues of the shortest-lived and warm-acclimated Kiel Bay mud clams, whereas lowest rates were observed in tissues of the longest-lived and cold-acclimated Iceland animals. In laboratory experiments with *A. islandica* from Kiel Bay, lipofuscin accumulation in tissues strongly increased at elevated seawater temperatures (E. Philipp, pers.

comm.), which confirms that ROS-induced protein- and lipid oxidation increases with increasing temperatures in the clams´ tissues.

Table 6.2: Environmental conditions at six sampling sites of *Arctica islandica* populations in the North East Atlantic and maximum life span potential (MLSP) of each population, modified after publication V. Mean annual temperature (T_{MA}) and salinity (S_{MA}) and their amplitudes (T_A, S_A) for each population were calculated from the ICES internet database http://www.ices.dk by Larisa Basova; Anthrop. Impact = anthropogenic impact, $Feeding_{MS}$ = Main annual feeding season. [1]Life spans data sources: Epplé et al. 2006; Wanamaker et al. 2008, Begum et al. 2010, publication IV and V. [2]Sources for regional anthropogenic impact = Arntz and Weber 1970, Pearson et al. 1985, Pihl 1994, Witbaard and Klein 1994, Nielsen and Richardson 1996, Rijinsdorp and van Leeuwen 1996, Witbaard et al. 1999; Rumohr and Kujawski 2000, Elmgren 2001, Eide and Heen 2001, Holte and Oug 1996, Wiltshire and Manly 2004, Kaiser et al. 2006. [3]Sources for main annual feeding season: Smetacek 1985, Lutter et al. 1989, Richardson and Christoffersen 1991, Gudmundsson 1998, Kosobokova 1999, http://www.bsh.de/de/Meeresdaten/Beobachtungen/MURSYS-457 Umweltreportsystem/Mursys031/seiten/noph2_01.jsp.

Population	Location	MLSP[1]	T_{MA} (°C)	T_A (°C)	S_{MA} (PSU)	S_A (PSU)	Anthrop. Impact[2]	$Feeding_{MS}$[3]
Kiel Bay	54°32´N 10°42´ E	40	7,3	9,3	20-25	4,8	high: eutrophication, fishery	March-September
German Bight	54°09´N 07°47´E	150	9,3	11,6	33	1,1	high: eutrophi-cation, fishery, increasing T	April-August
Norwegian Sea	69°39´N 18°57´E	93	6,4	5,6	34	0,7	organic enrichment, fishery	April-June
Kattegat	56°10´N 11°48´E	71	8,0	6,7	32	2,5	high: eutrophication	April-August
White Sea	66°18´N 33°38´E	53	3,7	12,6	26	2,7	low	May-August
Iceland	66°02´N 14°51´W	407	4,1	6,2	34	0,4	lower: fishery	April-June

Genetic variation between the six *A. islandica* populations from the NE Atlantic was found to be lower than the genetic variability within each population, which suggests that the different life expectancies represent phenotypic plasticity in *A. islandica.* The long duration of the planktonic larval stage in *A. islandica* which lasts between 30 and 60 days (Lutz et al. 1982; Mann and Wolf 1983) allows for wide dispersal of the larvae by ocean currents and is assumed to promote gene

flow between populations and a high level of heterozygosity within the populations (Dahlgren et al. 2000). Genetic variation is essential for the adaptability of a population to heterogeneous environmental conditions (Booy et al. 2000). Reed and Frankham (2003) stated that heterozygosity, population size, and quantitative genetic variation are positively and significantly correlated with adaptive population fitness. Thus, high genetic variability apparently enables the ocean quahog to successfully adapt to distinct environmental conditions in the Northern Atlantic Ocean. Phenotypic adjustments in response to fluctuating environmental conditions probably caused the development of different life strategies and life history traits within geographically separated populations of *A. islandica*. Highest differences in environmental conditions were found around Iceland and in the Kiel Bay, where *A. islandica* is a dominant member of the benthic infauna with high recruitment success in both regions (Brey et al. 1990; Ragnarsson and Thorarinsdóttier 2002). But while Iceland *A. islandica* are characterized by a long MLSP, low metabolic rates and late investment into reproduction (onset of reproduction between 10 to 30 years, Thorarinsdóttier and Steingrimsson 2000), shorter-lived Kiel Bay mud clams perform higher metabolic rates, higher cellular damage accumulation over age and maybe onset of reproduction at younger ages. Such evolutionary shifts are likely to occur when high and early adult mortality select for individuals that develop more rapidly and reproduce at an earlier age (Reznick et al. 2001a). Reznick and co-authors (2001b) investigated the population biology of guppies from seven high- and low-predation localities. Similar to salinity stressed *A. islandica* in the Kiel Bay, those guppies that suffered stress through predation pressure remained small in body size, produced offspring at an earlier age and were found to allocate more resources to reproduction than did guppies from low-predation environments (Reznick et al. 2001a; Reznick et al. 2004).

6.4. Reproduction and longevity in *A. islandica.*

Reproductive senescence is defined as a reduced reproduction success with ongoing age, caused either by a decline in quantity and/or quality of gametes production (Ridgway and Richardson 2010). On the contrary, the absence of senescence can be defined as ‘no observable decline in reproduction rate (= no reproductive senescence) or increased mortality after maturation ’ as well as ‘no age-related decline in physiological performance or disease resistance (= no physiological senescence)’ (Finch and Austad 2001).

To date, age related fecundity patterns in *A. islandica* are poorly explored. Jones (1980) reported that structure and development of gonads in 100 year old mud clams appeared to be identical to 20 year old specimens, and 100 % maturity was reported for the biggest size classes of a Canadian *A. islandica* stock (DFO 2007). Thus, there seems to be no indication of reproductive senescence in the older size or age classes of the ocean quahog, but at the same time there is no evidence that fecundity (= potential egg production by female) increases with age in *A. islandica* (Ridgway and Richardson 2010). In the literature, it is widely accepted that animals with infinite growth, such as fish, amphibians and mollusks increase their reproductive output throughout their lifetime due to age-related changes in energy allocation (Charnov et al. 2001), which does not

seem to hold true for the long-lived ocean quahog. Likewise, physiological fitness and reproductive capacities did not change over age in the long-lived red sea urchin *Strongylocentrotus franciscanus* (Ebert 2008) which reaches individual ages of > 100 years (Ebert and Southon 2003).
In contrast, an age-dependent decline in reproductive activity was visible in the short-lived mussel *Mytilus edulis* (MLSP = 24 years, Powell and Cummins 1985) ranging from the age of maturity (2 years) to the maximum recorded age of 10 years (Sukhotin and Flyachinskaya 2009). According to the mussel's gonadosomatic index (=fraction of gonad tissue mass in total soft tissue mass), individual fecundity as well as the proportion of normally developing embryos decreased over age (Sukhotin and Flyachinskaya 2009). In addition, a similar size-dependent decline in gonadosomatic index has been observed in the Iceland scallop *Chlamys islandica* (Vahl 1985).

According to Finch and Austad (2001) negligible senescence in *A. islandica* seems to include physiological and reproductive fitness over age. But the question arises: What is the evolutionary significance of such an extraordinarily long lifespan in the ocean quahog? Kirkwood and Austad (2000) showed that environmental or genetic manipulations that confer increased longevity in classical model organism for aging research (e.g. fruit flies, nematodes and mice) also confer increased resistance to environmentally imposed stressors. *Arctica islandica* is extremely adaptive to different environmental conditions and resistant to stressors such as strong annual fluctuations in salinity and temperature as well as to prolonged environmental hypoxia and anoxia (Oeschger 1990, Oeschger and Storey 1993, Diaz and Rosenberg 1995, publication V). I therefore hypothesize that longevity in *A. islandica* is an evolutionary by-product of its energy-saving life-style combined with constant investment into cellular protective and repair mechanisms and high resistance to elevated stress levels. Further, extrinsic mortality caused by predation pressure or fisheries impacts decreases with increasing age and shell size (= shell thickness) in the mud clams (Brey et al. 1990, Witbaard and Klein 1994). Therefore, *A. islandica* may reach individual ages of more than 400 years and successfully reproductive even in old ages when inhabiting protected areas with optimal environmental conditions such as those found along Northwestern coasts of Iceland. This is in agreement with Vaupel et al. (2004), who hypothesized that negative senescence is possible in organisms with indeterminate growth for example trees, but also fishes, reptiles, amphibians, mollusks and some echinoderms. Negative senescence is characterized by a decline in mortality with increasing age after reproductive maturity and undiminished reproductive capacity over age and is favored in species with strong repair or rejuvenative capabilities (Vaupel et al. 2004).

7. CONCLUSIONS AND PERSPECTIVES

In the present study, behavioral and physiological parameters were investigated in geographically separated populations of *A. islandica*. The overall goal of this work was to identify mechanisms and strategies that lead to the extraordinarily high maximum lifespan potential (MLSP) of the ocean quahog, and to examine the modulating effects of fluctuating environmental conditions on these strategies. For an inter-species comparison, physiological parameters were determined not only in the long-lived mud-burrowing clam *A. islandica* but also in the short-lived swimming scallop *A. opercularis*.

The results from this project suggest that the combined effect of different physiological adaptations and mechanisms favor extremely long individual life spans of up to several hundred years in the ocean quahog. These mechanisms are accompanied by very low but constant investments of energy over their life time when compared to shorter-lived bivalve species, where high amounts of energy are invested. *Arctica islandica* is characterized by slow growth rates, late sexual maturity, an inactive lifestyle and budgets its life time energy investments between somatic growth and maintenance and persistent but slow rates of gametogenesis.

Generally, low oxidative damage and constant investment into cellular protection and maintenance seem to extend single-cell lifetimes and to slow down the overall process of physiological aging in *A. islandica*:

I. Due to low standard metabolic rates (SMR), the formation rate of mitochondrial reactive oxygen species (ROS) is low in *A. islandica* when compared to other shorter-lived bivalves. These low metabolic rates are a consequence of low internal PO_2 in mantle cavity water < 5 kPa, which are maintained in spite of normoxic environmental conditions. As cells and, in fact, the entire animals are oxyconform, cellular oxygen consumption and ROS formation in isolated gill tissue were dramatically reduced at PO_2 of 5 kPa compared to 21 kPa. Together with a well-established antioxidant defense system, the low ROS levels represent strong arguments for extended longevity in *A. islandica* and, thus, for the validity of the ‘Rate of living theory’ (Pearl 1928) and the ‘Free radical theory of aging’ (Harmann 1956).

II. Metabolic rate depression (MRD) seems to be one of the key energy-saving and life-prolonging strategies in *A. islandica.* In contrast to findings in other vertebrates and invertebrates, dormant states in the ocean quahog seem to be triggered not only by external parameters (i.e. seawater temperature and food availability) but also by internal mechanisms. Avoidance of ROS-formation upon reoxygenation seems to be a protective mechanism in *A. islandica* dealing with the potential problem of strongly fluctuating internal PO_2 associated with burrowing, shell closure and surfacing. During MRD, when internal PO_2 decreases to 0 kPa, ROS production supposedly subsides as cellular respiration stops. The onset of anaerobiosis in the adductor mus-

cle of *A. islandica*, detected the earliest after 3.5 days of anoxic incubation or burrowing, is timed comparable late when compared to other bivalves. This limits the accumulation of acidic anaerobic metabolites in cells and tissues and curtails the needs for enhanced recovery respiration during surfacing. No ROS-burst was observed in isolated gill tissue of the clams following hypoxia-reoxygenation. Accordingly, neither the levels of enzymatic antioxidants (= activity of superoxide dismutase and catalase) nor those of low molecular antioxidants (specific glutathione content) were enhanced in tissues of *A. islandica* after 3.5 days of self-induced or forced MRD which would prepare for an oxidative burst.

III. The signaling molecule nitric oxide (NO) may play an important role at the onset of MRD in the ocean quahog during burrowing and shell-closure by inhibiting cytochome-*c*-oxidase as mantle cavity water oxygenation declines. Respiration in isolated *A. islandica* gills was completely inhibited by artificially produced NO at low experimental PO_2 with values ≤ 10 kPa.

IV. The preservation of high cellular defense and repair mechanisms over age as well as efficient mechanisms to remove and replace damaged cells seem to be crucially important for a high MLSP in *A. islandica.* Stable antioxidant protection over 200 years of age combined with low but constant cell turnover rates (= proliferation rates and apoptosis intensities) over 140 years of age in almost all tissues, are most likely responsible for reduction of cellular oxidative damage and slow senescence in the ocean quahog. Accordingly, protein carbonyle and lipofuscin accumulation proceeds far slower in tissues of the ocean quahog when compared to other bivalve species.

V. The only symptoms of old age ever found in the ocean quahog were decreasing cell turnover rates in the heart muscle over a period of 140 years. On one hand, this may indicate higher damage levels and possibly commencing loss in the functioning of the heart of aging *A. islandica.* On the other hand, decreasing cell turnover rates may indicate lower rates of waste product accumulation (e.g. lipofuscin, protein-carbonyles, DNA-damage) and cell damage in the heart tissue of *A. islandica* over lifetime and, thus, a reduced need for cell renewal.

VI. In the intra-species comparison, environmental adaptations rather than genetic variability seem to cause differences in MLSPs of geographically separated populations of *A. islandica*. Differences in sea water temperature, salinity, food availability and anthropogenic impacts are among the main environmental parameters determining MLSPs in *A. islandica* from different geographical regions.

VII. The scallop *A. opercularis* with high-energy throughput over life time, reduced investment into antioxidant defense, and early 'sig*ns of wear and tear*' represents a model bivalve species with opposite life and aging strategies when compared to *A. islandica*. The short-lived scallop reaches a MLSP of only 8-10 years and is characterized by fast growth rates, an active lifestyle and high SMR. The scallops reach sexual maturity at young age and feature high annual peak investments

into gametogenesis. Cell turnover rates (= apoptosis intensities and proliferation rates) were far higher in all investigated tissues of *A. opercularis* when compared to *A. islandica.* Subsequently, this indicates higher rates of cell damage and waste accumulation (e.g. protein cabonyles, lipofuscin). In agreement with these findings, tissue-specific antioxidant capacities decrease over age in *A. opercularis.*

Despite the ongoing development of aging research in bivalves, there is still a lot of basic biological research to be undertaken in *A. islandica*. In this work, the only physiological changes over age were found in the ocean quahogs´ hearts. Presently, it is still unclear, whether decreasing cell turnover rates in the heart muscle of *A. islandica* over lifetime indicate higher damage levels and possibly ongoing loss of functioning, or if they indicate decreasing cellular damage and therefore less necessity for cell turnover. Future studies, therefore, should look into cellular viability and oxidative stress parameters such as protein- and DNA-damage in the heart tissue over age. Complementing the present ecological and physiological research with studies of tissue-specific telomerase activity and DNA-repair mechanisms over age in geographically separate *A. islandica* populations, would help to fill these gaps of knowledge.

Metabolic depression was identified as a life-prolonging mechanism in *A. islandica*. In order to determine whether MRD-frequency and –durations vary in mud clams living under different environmental conditions, laboratory based burrowing-experiments should be conducted at different temperatures, salinities and food availabilities. Cellular and molecular processes involved in the induction of MRD in *A. islandica* and in the avoidance of ROS-formation during surfacing and tissue-reoxygenation need to be investigated in detail. The signaling molecule NO is possibly involved in the cellular signaling pathways that induces MRD. A first indication for the existence of nitric oxide synthase (NOS)-like activity in hemocyte cells of *A. islandica* was obtained in the present study, but needs to be confirmed in thorough detail. Because of time limited access to laboratory facilities such as the ISO-NOP-electrode, NO-generation in hemocyte cells could only be measured in one sample. Thus, measurements with the NO-electrode, and in parallel with fluorescent NO-specific dyes should be repeated in future experiments to ascertain and to characterize NOS activity in *A. islandica*. Further, gene expression (EST banks) and genome analyses could confirm the presence of NOS-genes in *A. islandica*.

In order to confirm, that ROS production rates are extraordinarily low in *A. islandica* after MRD during tissue-reoxygenation, ROS-formation rates should be investigated additionally in shorter-lived bivalve species of similar lifestyle, i.e. in mud-burrowing clams, or in bivalves living in the intertidal, frequently exposed to variable environmental PO_2 and to prolonged oxygen deficiency.

The alternative oxidase may function in avoidance of ROS formation during reoxygenation in *A. islandica* and investigations on the expression and protein-level could confirm or reject this

hypothesis. It would further be interesting to investigate the possible protective role of NO (see discussion) during surfacing and tissue reoxygenation.

Another insufficiently explored field in *A. islandica* research is the age-dependent reproductive capacity. Future studies should not only focus on the absolute numbers of gametes produced by the ocean quahog over lifetime, but also on the effect of parent age on the viability of gametes and fertilization success. It would be promising to investigate the onset of sexual maturity and reproductive senescence in different populations of *A. islandica* in the NE Atlantik in order to approve or reject the hypothesis that clams of shorter MLSP, which suffer higher environmental stress (e.g. Kiel Bight, White Sea) produce at earlier age and allocate more resources into reproduction than do clams from low-stress environments (e. g. Iceland).

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Et hätt noch immer jot jejange (Kölsches Grundgesetzt, Artikel 3)

Eidesstattliche Erklärung

(Gem. § 6(5) Nr. 1-3 PromO)

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Bremen, 02.März.2011

Julia Strahl